

Mr Gromyko and Mr Reagan

Last week's meeting in Washington seems to have something to do with future talks on arms control, but nobody knows quite what. The best hope is that Mr Reagan will redefine his policy.

MR Andrei Gromyko caused such a stir on his visit to Washington last week that observers could have been forgiven for thinking him a candidate for the US presidency. On the face of things, he has many of the essential attributes. He has been around for a long time and he is also an elderly gentleman. But his looks belie his age, suggesting that the longevity that comes with the successful exercise of power is on his side. He is also capable of those mercurial changes of mood that, in senior politicians, pass for inscrutability; a tough hardliner's speech at the United Nations on the Tuesday, an exit from the White House with his hands clasped in the traditional boxer's gesture of self-congratulation on the Thursday. By popping in and out of the United States like this, Mr Gromyko has cleverly changed the agenda of speculation from mundane questions such as whether interest rates will rise still further (and, if so, before or after the election) to simpler issues — what did he say to the real president (who is not quite as old), and in what tone of voice?

We shall have to wait until after the election to find out. It would have been no part of Mr Gromyko's purpose to help Mr Ronald Reagan to his re-election a month from now, for which reason it was never on the cards that a tangible agreement on anything would have emerged from last month's meeting. The Soviet Union has nothing to lose, and everything to gain, from postponing serious talk about East-West relations until after the election. But there was also, last week, much to be gained from emphasizing in the run-up to election-day the importance that the Soviet Union attaches to what politicians in the West may be tempted to say in the heat of an election campaign. Mr Gromyko, at least, will not have forgotten that President Reagan's previous election campaign was marked by repeated declarations that arms control negotiations then negotiated, Salt II in particular, were Soviet traps for the unwary. In the event, the first Reagan Administration took two years to learn that that position was untenable. Nothing could have been lost, and everything gained, by reminding the incumbent president of that waste of everybody's time and of the need to avoid a repetition of that error.

It is also in everybody's interest that the momentum of arms control negotiations should pick up again once the election result is known. On the assumption that Mr Reagan will not be a lame-duck president waiting for a successor to take office, the fallow period before the next inauguration day could be used constructively, especially if Mr Reagan should let slip during the election some strictly verbal promise likely to appeal to Mr Gromyko and his fellow-countrymen (not to mention many of Mr Reagan's). An undertaking that a moratorium on the testing of anti-satellite weapons would be a precondition of negotiations on these instruments would suit capitally. (Mr Reagan will have had a chance to let that carrot show in his speech this week to the United Nations.) The temptation to seem conciliatory must be huge. There will be no complaint if Mr Reagan should succumb to it.

Preparing the ground for negotiations on anti-satellite weapons is not however what matters most. Both Mr Reagan and Mr Gromyko should remember that. For practical purposes, the agenda for arms control in the next few years should not be determined by the imagined anxieties of statesmen on either side. The US Administration's ambitions for a star wars defensive system are not an immediate threat to stability, but a threat to the anti-ballistic missile treaty (part of the Salt process) ten years from now, a threat to stability twenty or thirty years from now — and a threat to everybody's pocket-book in the meantime. The immediate issues, however, have been what they have been for ten years — the needless investment in missile systems on both sides, their fluctuating imbalance and needless pieces of irrationality such as chemical weapons. The United States is right to have been insisting that these are the subjects crying for discussion.

Can the present administration, or a successor in the same mould, bring itself to consider these questions constructively? Mr Gromyko may also have been looking in Washington for guidance on that point. Nobody can say for sure, and indeed in the past few months, there have been disconcerting signs that too many senior officials in Washington are convinced (entirely against experience) that agreements with the Soviet Union are not worth the paper they are written on. That mood, or those people, will have to be exorcised if ever there is to be serious negotiation on arms control measures that will count. Fortunately, the election provides the president with an opportunity to redefine the basis for negotiations without spilling the blood of those who have helped him loyally in the past four years. The perennial problem of politicians running for office, that of making too many speeches with too little material, might be deftly solved by using an occasional speech to lead the White House out of its old-fashioned xenophobia. It would be ironical if Mr Gromyko had been putting Mr Reagan up to that old democratic trick. □

Nature and the Soviet Union

ON 22 November, *Nature* will publish a survey of science in the Soviet Union.

When the project was first planned, early in 1983, it was hoped to visit a number of institutes and laboratories in the Soviet Union, but repeated requests for assistance have now been met with the information that arrangements cannot be made until 1985. Accordingly, *Nature* would be glad to hear from scientists with recent first-hand experience of work in the Soviet Union, or of collaboration with Soviet scientists and/or institutes.

Those able to help are asked to write to the editor as soon as possible at one of the editorial offices in London, Tokyo and Washington. Please include a telephone number.

Anonymous correspondence will not be dealt with. All correspondence will be confidential unless otherwise agreed. Information will not be published without corroboration. On this occasion, *Nature* is exclusively concerned with content and texture of Soviet research, not with problems of dissent and emigration. □

Another culprit

The largest of the British research councils is not blameless for the plight of British research.

BRITISH self-contentment over the mechanism of research support has taken several hard knocks in the past few years, nowhere more conspicuously than over what is called the dual-support system, the mechanism by which public funds for academic research are channelled from two supposedly



independent sources, the University Grants Committee (which is supposed to equip university departments to carry out research) and the five research councils (which then provide project funds on a selective basis). That the system has not been working as intended has been clear at least since the 1970s, although people differ in their diagnoses of what is wrong. Two years ago, the Merrison committee (commissioned jointly by the research councils and the grants committee) put most of the blame on the universities, but last month's forward strategy published by the University Grants Committee proudly took credit, on behalf of the universities, for roughly two-thirds of the cost of academic research with British public funds. The issue of which has been the weaker link is bound to be influential in the next few months, when the British Government will have to respond both to the grants committee's strategy document (probably early in 1985) and, more immediately, to the research councils' appeal (now lodged) for extra funds.

So where should the blame lie? The universities have been lackadaisical offenders for several years, adjusting to shrinking budgets by skimping on discretionary expenditure (among which research expenditure has been conspicuous), while the general increase of teaching loads casts doubt on the grants committee's assumption that something like 30 per cent of the university salary bill is a direct contribution to research. But the faults are not all on one side. The research councils are in their separate ways vulnerable to a variety of complaints, of which the most familiar is that so much of what they have to spend is committed to their own research institutes that they have too little to spend on newly arising projects. The research council responsible for the underlying welfare of British agriculture has been the most consistently pilloried on this count, but perhaps unfairly — many of its institutes are legacies from the distant past, not merely from the time, fifteen years ago, when prudence required that research councils should keep their fixed obligations to a minimum for the sake of flexibility. In any case, this line of defence continues, agencies with responsibility for research which is strategic in character (in Britain, medicine, the natural environment as well as agriculture) must seek some measure of continuity by institutionalizing parts of their programmes. The result, however, is the now notorious difficulty of effecting change. The problem for managers in the months ahead is to create room for manoeuvre.

Headhunting

In case there are doubts about the need, the curious circumstances of the British Government's difficulty in recruiting able people as heads of the research councils is a telling reminder of malaise. The post of chairman of the Natural Environment Research Council has been filled on a caretaker basis after one person to whom the job was offered stipulated "impossible conditions" — no doubt another way of referring to a demand for assurance about the council's future prospects and its chairman's freedom of action. The vacancy at the agricultural council, which has been put out to headhunters, is yet another sign that working researchers of distinction are unwilling to turn their backs on active science so as to administer the funds that help to keep other people active. The disincentive is that both research councils' funds are too meagre to serve their stated purposes and that an applicant for one of the top jobs cannot know in advance whether some of what there seems to be to spend will be snatched away from one year to the next.

This dilemma will be further sharpened in the next few months, when the government will be required to find a successor to Dr John Kingman, the chairman of the Science and Engineering Research Council who will be leaving less than a year from now to become vice-chancellor of the University of Bristol. On the face of things, this is the most attractive of all the top jobs. The council is the largest of all, with an annual budget equal to all the others put together. Moreover, this council differs from the others in that its terms of reference are explicitly to further the cause of academic science (which is why the council was not directly constrained by the Rothschild reorganization of 1973). Yet even this huge organization is now almost as hamstrung as the others. Only a few months ago, one of the council's own sub-committee's

was wringing its hands over the tiny uncommitted sums of money available for spending on what was called "core" research — research in the physical sciences not tied to one of the council's national programmes of research in high-energy physics, astronomy, information technology or what have you. The difference is that while the strategically-orientated research councils can plead that their inflexibility has been wished upon them by historical accidents, the Science and Engineering Research Council has wished its problems on itself.

Costly trends

Over the years, several tendencies have been mistakenly allowed to perpetuate themselves, not the least of which is the growth of the share of the council's budget spent on central facilities ostensibly intended as services to academic science. The cost of the annual subscription to the CERN laboratory is the most conspicuous (and recently, the most contentious) of these costs, but is a declining proportion of the whole. Other facilities, such as the neutron spallation source in Berkshire (due to come on stream in November, six months late) are modest in scale (£18 million in capital cost) but justifiable against the background of possible cancellation chiefly by the argument (as at a recent council meeting) that completion would then cost next to nothing, and thus yield the cheapest machine of its kind anywhere in the world. The council's weakness for bricks, mortar and institutions is similarly evident; its agonized consideration a year ago of the question whether there should be two major observatories in Britain (Greenwich, Edinburgh) ending with the decision to keep both (as well as a castle near the Sussex coast). In recent years, the council seems to have found it difficult to make decisions.

Matters are further complicated by the council's way of dealing with the funds it has to spend. Everybody acknowledges that spending £250 million a year is difficult, but that simple consideration does not fully account for the council's predilection for distributing spending authority to four powerful committees (called boards) rolling logs for their own special interests. Weakly, the council has allowed (even encouraged) the growth of the board responsible for engineering research without providing much in the way of objective evidence that the pattern of academic engineering research resembles in any important way that common in other fields; the most charitable explanation is that engineering is politically popular (and that engineers are a turbulent group). It is especially iniquitous that the council should have parcelled out to its spending boards responsibility for the allocation of research studentships, the stipends by which graduate students are supported in Britain. The consequence is that by the allocation of research studentships to particular university departments (in whose gift their ultimate award to students rests), the boards are able to insulate themselves from suspicions that young people's estimation of what is interesting may be different from their own. In recent years, the council as a whole has further reduced its own freedom of manoeuvre by its willingness to set up earmarked funds (trendily called "directorates") to spend money on fashionable causes, biotechnology or information technology, as the fashion strikes.

To be fair, there have recently been signs that the council has occasionally seen the error of its ways. Within the past year, Dr Kingman has tried (and failed) to combine the two most powerful of his boards (nuclear physics and astronomy), hoping in the process that similar criteria would have applied in the two very different fields. It is now also firm council policy that the proportion of the council's budget spent on central facilities will in future decline from its peak (in 1982–83) of 37 per cent. There is no sign of repentance on the way in which research studentships are allocated, but that may yet come. In all this, the council's strongest defence is that even its comparatively large budget is far too small to accomplish everything it is supposed to do, and there is force in that claim. All the more reason, however, why the council should be more vigilant than in the past in creating some flexibility within the straitjacket it has made for itself. But that will be a thankless task, hardly the kind of job which people will be flocking to secure. □

European synchrotron source

Strasbourg and Grenoble battle for site

THE European Synchrotron Radiation Source (ESRS), a world-rank 5 GeV electron synchrotron first planned and championed in the late 1970s by the European Science Foundation (ESF), seems likely to be built at last, at Strasbourg on the Franco-German border. Or so the auguries suggested last week, in the midst of an extraordinary political and "scientific" battle now raging between the little tourist town of Strasbourg and the French alpine city of Grenoble, both potential sites for ESRS.

The battle is all the more extraordinary after the years ESRS has spent in the wilderness. The idea for an intense next-generation synchrotron radiation source was first canvassed in 1979, when ESF (a consortium of European research councils) began touting its technical study around Europe — without success. But now, partly as the result of a poll showing a potential ESRS user community of 2,000 scientists and 600 research groups in Europe, and much interest in industry, Cinderella has become the belle of the ball.

In the latest move, the French and German research ministers have reached a deal that will place ESRS in France and (as compensation) a "European trans-sonic wind tunnel" in Cologne, West Germany. (Britain was originally in the running for ESRS, but dropped out because of the high expenditure expected of successful bidders.)

Now, with the main battle settled in favour of France, the two regional governments that might benefit have raised the stakes by throwing money (and glossy brochures) at the project. Strasbourg, the home of ESF, has bid FF 100 million (£85 million) to win the project, and Grenoble has offered FF 80 million (£65 million) towards the FF 900 million (£750 million) cost of the machine. If ESRS were built at Grenoble, it would go alongside the high-flux neutron beam facility, the Institut Laue-Langevin (ILL), which is insisting that the "scientific case" would favour such a link.

French regional, economic and scientific policy, however, favours Strasbourg. And this is a powerful force in Paris at present, with the government seeking to decentralize and to create new regional centres of excellence. Strasbourg is also more centrally located in Europe, which is why West Germany favours it. Although Britain would prefer Grenoble, its lack of cash gives it no leverage. The choice now rests with the French Government. Paris sources say that Strasbourg is the likely but not certain bet in a decision that should be taken in a few weeks.

On the scientific side, the battle for ESRS appears at first sight to be between the biologists (in whose discipline the University of Strasbourg and surrounding institutes excel) and the physicists (where Grenoble is on a level with Paris).

But do the biologists want the machine? Some say yes, some say no. The directorate of the major French research council, the Centre National de la Recherche Scientifique (CNRS), wants ESRS for the biologists and so favours Strasbourg, where CNRS already runs a centre for low-energy nuclear physics and a number of important laboratories in molecular biology and where it plans a major research centre in plant biology. Many individual CNRS biologists, however, have a different view: they would prefer that ESRS stayed with the physicists in Grenoble.

According to CNRS, a Strasbourg location for ESRS would help biologists (and chemists) to become more involved with the use of synchrotron radiation and break down interdisciplinary barriers. It

would also contribute to the development of Strasbourg as a centre of European science, close to West Germany and Swiss centres. CNRS biologists are worried about having to help pay for a facility that in practice they will not use. They say their budget within CNRS is already tight, burdened as it is with more than their share of an existing French synchrotron source (LURE) at Orsay, whence their preference for Grenoble.

The director of the European Molecular Biology Laboratory (EMBL) at Heidelberg is said also to be in favour of Grenoble, where EMBL has a neutron scattering facility. ILL's own case is that its own work — the instrumentation as well as scientific interests — is similar but complementary to that of ESRS, so that a Grenoble site for ESRS (or "Maxwell Institute" as ILL has already dubbed it) would be more productive than at Strasbourg. ILL says there is room (just) on its compact site for the 772-metre-circumference synchrotron, and that vibration from the nearby auto-route should not be the nuisance once feared.

Grenoble's case is strong, but Strasbourg has politics on its side. No wonder that the French science minister, Hubert Curien, says that the decision "is not proving easy".

Robert Walgate

Marinov

Further threat of immolation

BRITISH diplomatic missions in Genoa (Italy) and Vienna (Austria) have been under siege in the past few weeks by Dr Stefan Marinov, the Bulgarian physicist now living in the West, with threats of self-immolation. Dr Marinov is protesting at the refusal of the Editor of *Nature* to publish three long scientific articles, one of which is a restatement of Marinov's theory of absolute space-time, another of which announces the design of a *perpetuum mobile*.

Dr Marinov first embarked on self-immolation in Genoa on 8 August, outside the British Consulate, but by his own account was held in conversation for half an hour by staff from the consulate and was compelled to flee when the staff sought the assistance of the police, knowing that he was present illegally in Italy (having been denied entry in 1980).

At a press conference held in Austria last week, Dr Marinov again announced his plan for self-immolation, this time outside the British Embassy in Vienna. On the telephone earlier this week, he said that he would take this step at 10 a.m. local time on 2 October but, on being informed of the appearance of this piece, said "Now I don't know whether to immolate myself tomorrow".

Dr Marinov's claims that Einstein's theory of special relativity is misplaced were first made in 1974 (*Czechoslovak J.*

Phys. B24, 965; 1974), on the basis of an experiment carried out in Bulgaria that purported to show that the velocity of light is direction-dependent. Marinov has since claimed to have repeated the experiment. The paper describing that investigation, "New measurement of the Earth's absolute velocity with the help of the coupled shutters experiment", says that the experiment was carried out in his girlfriend's apartment, that the rotating shutters, functioning as a siren, disturbed the neighbours and that "after a couple of altercations, my girl-friend threw away from her apartment not only my apparatus but also me".

The two other papers submitted for publication are called "On the action and interaction of stationary currents" and "Coup de grace to special relativity and to something else". All three papers have now been published by Marinov in his book *The Thorny Way to Truth* (Part II).

Marinov's complaint against *Nature* is primarily that publication of these three papers has been refused, but also that "milliards of dollars" are being lost each day the world remains ignorant of his perpetual motion machine. Other matters in dispute are *Nature's* refusal to publish an appeal to the late Yuri Andropov at the end of last year and, earlier, a manifesto called the "Ten Jena Commandments".

John Maddox

Canadian science

New brooms plan clean sweep

Ottawa

CANADA's science chiefs are expecting great things from their new progressive conservative minister, Thomas Siddon. With a professional engineering background and a PhD in aero-acoustics, Siddon is highly spoken of as a friend of science and may be the man to gain some much-needed political clout for his office as minister of state for science and technology.

One of the first signs of renewed emphasis on science may be the creation of a standing parliamentary committee to deal with technology development. And by all accounts the recent Wright Task Force report urging more demand-led research and development (see below) is being taken very seriously. While some science administrators admit to private doubts about the numbers, Siddon intends to deliver his party's pledge to double Canadian research and development to C\$ 10,600 million within its first term of office.

Most of this increase needs to take place within industry. Canada suffers from being a "branch plant" economy — many companies based in the United States use Canada simply for warehousing and distribution — and this Siddon is determined to change. The activities of major companies will be examined and government investment and tax measures deployed to favour more innovation within the country. There is a strong feeling that trading competitors have been making gains from various forms of hidden assistance to their domestic industries, and the intention now is that Canada should use every fiscal measure permitted under the General Agreement on Tariffs and Trade to protect its home base.

One high priority is to revise the scientific research tax credits introduced earlier this year. That scheme, intended to help young high-technology companies that did not benefit from traditional tax incentives, has already proved an embarrassment: although a boon for investment dealers, it has cost the government significant revenue and has failed to generate innovation on the scale intended.

Changes can be expected at laboratories run by government departments and by the National Research Council (NRC), which cover everything from measurement standards to cancer research. A proposed manufacturing technology centre to be run by NRC was planned by Wright as representing "everything that is wrong with federal technology policy": industry was not consulted and the facility may be redundant before it is built. NRC's technology transfer programme has failed to make a real impact on Canadian industry, and Siddon wants to contract out

some NRC work to make more productive use of the laboratories.

While most Canadian universities have science departments, subject cover is patchy and there is serious concern about the future supply of highly qualified manpower. Despite Siddon's obvious enthusiasm for development as opposed to research, he "can give an assurance" that federal support for university-based

Looking south

THE Canadian economy is beset with problems stemming from its historical reliance on natural resources and its poorly-developed indigenous industry: more than 50 per cent of manufacturing industry is foreign-owned, and the per capita budget deficit is much larger than that in the United States. Research and development accounts for only 1.28 per cent of the Gross National Product, less than half the figure in the United States, and by the latest unofficial estimates the proportion is actually decreasing.

One major difficulty is the lack of venture capital: unlike their entrepreneurial neighbours to the south, Canadians prefer to save their money than to put it at risk, and insurance is big business. Many banks refuse to make loans for commercial ventures unless the idea has previously been tried in the United States. Canada also lacks the defence-based industries of the United States.

A hard-hitting task force report released in July urged the government to stimulate private sector research by rationalizing and simplifying industrial support schemes, providing better tax incentives and redirecting government procurement policies. The task force, under the chairmanship of Douglas Wright of the University of Waterloo, said the federal government should pay the full cost of university research, thus eliminating the political dimensions of the present research support system under which indirect research costs ("overheads") are met by provincial governments. Cooperation between universities and industry should be strengthened by a flat-rate grant to universities undertaking industrial research and development by tax concessions for industrial companies making use of universities.

Wright was highly critical of Canada's federal laboratories, some of which are "struggling to find appropriate challenges". The suggested solution was that directors recruited from industry should be given authority to set goals for federal laboratories, encouraging them to contract out research as much as possible. A formal government response to Wright is in preparation.

Tim Beardsley

research will be "continued and expanded". But this will require difficult negotiations with provincial governments, which, though supposed to support education, have in practice ensured a creeping increase in the unit cost of research supported by the research councils. Siddon also wants to encourage academics to follow through development of important discoveries by promoting the idea that consultative work for industry as well as peer-reviewed academic publications can advance a university career.

Much of this ambitious programme will depend on what sort of influence Siddon can wield in cabinet. There is room only for improvement: science and technology has at different times had to share a minister with other departments and there have been 10 incumbents of the office since 1971. But Siddon, who was a critic of science and technology in the last parliament, has 5 years' parliamentary experience, making him almost a veteran in the cabinet. The traditional hewers of wood and drawers of water had better watch out.

Tim Beardsley

Microscope acquired



ONE of the earliest British scientific instruments to be designed, constructed and marketed outside London has just been acquired by Edinburgh's Royal Scottish Museum. The instrument, a 2-inch high silver microscope made in 1749, was a product of the workshop of John Clark, an Edinburgh goldsmith, jeweller and instrument maker. Although its eighteenth century purchaser would have been a Scots gentleman of leisure, desiring a novel curiosity, it was nevertheless the patronage of such customers that spurred instrument makers to develop the skills they would need to meet the demands of their first scientific and medical customers. The museum was assisted in making the purchase by the National Heritage Memorial Fund.

European pollution

Towards lead-free auto fuel

Brussels

EUROPE is edging closer to a substantial reduction in exhaust pollution. But the ride has been far from smooth. Last week, the European Commission added the final flourish to proposals designed to phase lead out of petrol (gasoline). But only a few days earlier, West Germany announced its intention to apply US exhaust emission standards, which are twice as strict as those now in force in Europe, by 1989, rather than following the Commission's two-stage timetable.

With many of the 90 million cars on European roads expected to be running on super petrol by the early 1990s, the European Commission has ruled that the EEC countries should sell a single super-grade unleaded petrol from October 1989, although countries such as Italy and France, with a considerable market for small-engined cars, will still be able to sell a normal lower octane-rating unleaded petrol as well. (Unleaded petrol in Europe in fact means a lead content of between 0.01 and 0.02 grams per litre. The US content is 0.0135 grams per litre.)

Since both leaded and unleaded petrol will have to be available until old cars have been phased out, the super-leaded petrol will be marked with a red dye, to avoid mistakes in filling but also to make spot checks on "wilful misfuelling". The Commission feels price incentives should be encouraged, but this has so far been left to national governments.

Other technical specifications for the petrol (density, volatility, etc.) will be standardized by the European standards body, Le Comité Européen de Normalisation (CEN).

The proposals are the final touch to a series of European emission controls which environment ministers have to decide upon by the end of the year:

- Reducing the lead content of leaded petrol throughout the Community to 0.15 grams per litre, by 1989. Only two EEC countries, West Germany and Denmark, apply the minimum limit laid down by the 1978 EEC directive. But it is likely that the United Kingdom and the Netherlands will be allowed to impose that limit in 1986.
- Introducing unleaded petrol for new models of cars from 1989, and for all new cars sold from 1991 onwards.
- Imposing a limit of 5 per cent for benzene contained in the new petrol "cocktail" dictated by the removal of lead. Benzene is thought to be the source of a particularly toxic aromatic hydrocarbon in automobile exhausts. Some EEC countries would like the 5 per cent limit to apply to leaded petrol as well.
- Reducing other exhaust emissions (carbon monoxide, hydrocarbons, nitrous oxides) to Japanese and US standards in two stages by 1995.

Last June there appeared to be a consensus among environment ministers to adhere to the Commission's timetable. But more recently Denmark, Luxembourg and the Netherlands have seemed inclined to change to US standards in an earlier single move, and West Germany announced on 19 September that from 1 January 1989 new models of cars would have to be fitted with anti-pollution devices. The law would apply to 2-litre engines from 1988.

Whether West Germany will dare to go it alone is questionable, because of EEC rules on competition and free movement of goods. The European Commission is also doubtful about the tax incentives linked to the purchase of cars which are "environmentally friendly" (*Umweltfreundlich* in German), which could be seen as an unfair subsidy for the West German motor manufacturing industry.

While British and French car manufacturers have been quick to protest about unfair competition in Europe, West

Germany fears competition just as much from the Japanese, whose ready-made models already fitted with catalytic converters for the home and US markets will, they fear, invade the West German market.

Meanwhile, Chancellor Helmut Kohl has also been severely criticized by the opposition parties for bowing to the industrial lobby, and delaying West Germany's original move to lead-free petrol scheduled for 1986.

West Germany's unilateral intentions have made a breach in the European consensus and could slow down progress on emission control in Europe unless ministers can reach a compromise before their meeting on 6 December.

Once the decision on these emission controls is taken, perhaps attention will be directed to the 10 million lorries and the 10 per cent of private cars that run on diesel fuel.

In addition to other pollutants (but not lead), diesel engines spew carcinogenic unburnt particulates (smoke) into the air. So far neither the United States nor Japan has been able to solve that problem either.

Anna Lubinska

Lead and pollution

Brussels

TWENTY per cent of air pollutants such as carbon monoxide, nitrous oxides and hydrocarbons come from the exhausts of motor vehicles. Permitted emission levels have been lowered four times since the European Community started regulating exhaust emissions in 1970.

The latest proposal involves reducing emissions in two stages to the very strict US and Japanese levels. These are as follows:

	Japan (grams per km)	United States (grams per mile)
Hydrocarbons	0.39	0.41
Carbon monoxide	2.7	3.4
Nitrous oxides	0.48	1.0

The first stage — by 1989 — aims at reducing emissions of carbon monoxide by 20 to 50 per cent (depending on the type of vehicle), hydrocarbons and nitrous oxides (which together form smog) by 20 to 40 per cent, and nitrous oxides alone by 30 to 45 per cent. Although emission levels in diesel-run cars are also to be reduced, they

cannot be reduced to the same extent.

But the second stage, by 1995, requires the use of further technology. Although the development of lean-burn and fast-burn engines (which would produce less pollution in the first place) has been stepped up, the most efficient anti-emission pollution technology available today is the three-way catalytic converter. Fitted to the exhaust of a car, it chemically converts polluting exhaust emissions into the harmless components of the air we breathe: carbon dioxide, water, oxygen and nitrogen. Although research has been carried out into catalytic converters which have some tolerance of leaded petrol, their efficiency is limited.

Catalytic converters now in use are damaged by lead. Hence the need, even if lead were a doubtful health risk, to remove it from petrol. In order to fit "cats", which would reduce smog, in the early 1970s, the United States had to get rid of lead first. Europe has approached the problem from the opposite angle.

Anna Lubinska

Weinberg *et al.* threat from animal lib

"Researchers beware!" was one of the immediate and somewhat unexpected British responses to the *Nature* conference on the molecular biology of cancer, at Boston two weeks ago. Within a week of the meeting, a document was widely distributed to laboratories in Britain, insisting that the paper presented by R. Weinberg ("Neoplasms as uncontrolled regenerative hyperplasia") would produce "horrible tissue trauma and wound healing experimentation... that must be publicly

revealed, legally examined, morally questioned and humanely stopped".

Weinberg was accused of secretly communicating his "wretched and detestable experimentation" at a closed meeting rather than openly publishing it in the literature. How the Animal Liberation Front, the authors of the warning, intends to "punish those responsible" is unclear, but the organization claims that it will not tolerate the use of experimental animals in cancer research. □

ICSU

Grand plans for global changes

Ottawa

THE International Council of Scientific Unions (ICSU) last week approved in principle a major international interdisciplinary research programme on interactions of the geosphere and the biosphere. The aim of the programme is to understand global change over long time-scales, and in particular the influence of man on the environment.

Advocates of the venture, to be called the International Geosphere Biosphere Program (IGBP), argue that instruments now becoming available should be used in a coordinated fashion to monitor environmental changes that might affect human civilization as the Earth's population increases. Specific proposals are so far lacking; but the fate of carbon in the ocean and the long-term effects of high-yield agriculture on soils are likely to figure prominently.

But despite the laudable sentiments expressed last week by those seeking to avert possible environmental disaster, many were critical of what they saw as woolly thinking. Professor Tom Malone of the US National Academy of Sciences, a prominent champion of IGBP, promoted J. E. Lovelock's "Gaia" superorganism concept of the global environment, an idea most biologists find untenable. Speaker after speaker at a day-long symposium at last week's ICSU meeting emphasized the inter-relatedness of various facets of the geosphere and biosphere without specifying what new studies were needed.

Interestingly, some of the reactions heard last week may reflect the different styles of science in Europe and North America. One summary talk that featured a slide of starving children was privately criticized by some European delegates for using cheap tactics, while being praised for its effectiveness by American scientists. The second group argued that the purpose of the symposium was to stir delegates to action rather than to advance understanding.

The British Royal Society has been the leading opposition to IGBP, believing that unless it was more carefully focused, the project could divert attention, and possibly resources, from existing international studies such as the World Climate Research Program. Some of the ideas canvassed for IGBP last week were nothing if not ambitious, with up to 50 dedicated Earth satellites being proposed. A compromise put forward by Academician V.V. Belousov of the Soviet Union and agreed by the general assembly calls for an *ad hoc* committee to work out specific proposals for IGBP in time for the next ICSU general assembly in 2 years' time. Sir Arnold Burgen, foreign affairs secretary of the Royal Society, said he was "quite happy" with Belousov's formula.

Everybody stresses that the programme is unlikely to produce data before the 1990s, and that the sort of long-term data acquisition needed will require a shift of attitudes from short-term fashionable projects towards more methodical data collection. But the success of International Geophysical Year in the late 1950s is being held up as an example of what might be achieved.

Tim Beardsley

Visas denied

THE Ottawa meeting of ICSU was marred by angry exchanges and protests by the Soviet delegation at the denial of entry visas to two Soviet representatives. A statement by the Canadian Ministry of External Affairs read out at the meeting said the government had ensured visas for "all *bona fide* scientists" to facilitate free association.

The two excluded Soviets were Dr V. K. Dobrosel'skii and Mr A.A. Kokoshin. Dobrosel'skii is, ironically a member of ICSU's standing committee on the free circulation of scientists. He is also widely rumoured to hold the rank of brigadier in the KGB (the Soviet secret police) and has previously been denied entry to other Western countries. Soviet delegates, insisted however, that Dobrosel'skii has several "outstanding" inventions to his credit. There was more puzzlement over the exclusion of Kokoshin, who is a member of ICSU's committee on science and technology in developing countries.

Most found the exclusions surprising and regrettable. There is no obvious rationale for a political reprisal by the newly elected Canadian Government, and some observers from Western nations believe Dobrosel'skii has genuinely worked for free movement of scientists regardless of what other positions he may hold. Everyone recognizes a grey area — ICSU has never consisted solely of active research scientists — and, as one US delegate observed, "we've some good scientists in the CIA" (Central Intelligence Agency). The assembly passed a resolution expressing its "deep concern" that entry visas were still being denied to scientists attending international meetings, singling out the governments of India, the Soviet Union and Canada. ICSU-sponsored activities will not be held in these countries until solutions have been found.

Visa problems also explain the Indian National Science Academy's decision to withdraw its offer to host the next ICSU general assembly; it has apparently been unable to secure the guarantee required by ICSU that scientists would be allowed entry visas. The 1986 general assembly will instead be held in Switzerland, but India may still be the host in 1988. Tim Beardsley

Drugs in athletics

Mortality of Soviet athletes

SUSPICIONS that the Soviet Union, and possibly some other socialist-bloc countries, routinely use anabolic steroids and other biochemicals in the training of athletes have now been fanned by an underground report which reached the West at the end of July, just in time for the Los Angeles Olympic Games. The report lists the names of 59 former Soviet Olympic competitors (including 26 medallists) from the 1952 games onwards, who have since died. The list, it is claimed, was compiled with the participation of Soviet athletes, many of whom fear for their own life-expectancy as a result of training procedures.

Although the list includes a few cases of



accidental death not related to sports, the overall figures show a death rate of 17.1 per thousand competitors, whereas the corresponding figure for the United States over the same period is 4.5 per 1,000 and for the United Kingdom 2.1 per 1,000. Deaths occurred most frequently in the 41–50 age group (18), 31–40 (11) and under 30 (9). The death rate, according to these figures, has more than doubled since 1976, while remaining stable or even decreasing elsewhere.

The conclusion that these figures are related in some way to specifically Soviet training practices has been challenged by Michael Kessis, a physical education instructor in California who edits the *Soviet Sports Review* (a collection of translations from the Soviet sports press), but not, so far, by the Soviets themselves.

Thus, in a comment on "anti-Soviet" attitude in Los Angeles, *Literaturnaya Gazeta* did not deny the deaths but merely pointed out that Vsevolod Bobrov, the hockey-player, had been "approaching 60" when he died (he was in fact 57) and that Valerii Kharlamov (another hockey-player) and Valerii Popenchenko (a boxer) had died in "tragic accidents".

Apart from accidental victims, the

deceased athletes received no obituaries in the Soviet media, but the TASS correspondent in New York, Vladimir Kikilo, was able to confirm a number of the deaths. Given the uncertain provenance of the document, it would otherwise have been necessary to check its contents by a scan of the Soviet sporting press for the last

Mortality of Olympic medallists (1952–76*) by age group at time of death

Age	Soviet Union	United States	Germany (FRG & GDR)**
30 and under	9	7	3
31–40	11	1	3
41–50	18	4	4
51–60	7	1	2
61+	1	1	0
Total deaths	46	14	12
Those dying in 1976–82	26	3	4
Total medallists	1,033	784	786
%Deaths	4.45	1.79	1.53

*The 1980 Moscow games are omitted from this survey due to the US boycott.

**Figures amalgamated because in 1952 East Germany did not compete and from 1956 to 1964 the two Germanies fielded a joint squad.

recorded appearance of the listed competitors.

The ban on the use of anabolic steroids for athletes makes it difficult to obtain statistically significant data on their long-term effects. Earlier this year, however, *The American Journal of Sports Medicine* listed no fewer than twelve steroid-related disorders, ranging from testicular atrophy in males and virilization in females to hepatic dysfunction, hypertension, changes in cholesterol composition, tumours, changes in connective tissue and psychosis.

There have also been suggestions that the Soviets may be administering to their athletes carnitine (involved in fatty acid metabolism), potassium orotate (an intermediary in the biosynthesis of pyrimidine nucleotides) and inosine (also involved in pyrimidine nucleotide synthesis and in the formation and breakdown of high-energy phosphates). Such compounds could serve both as performance enhancers and to counteract the short-term side effects of steroids. The long-term effects of high dosages are, however, unknown.

The mortality report on Soviet athletes — if accepted abroad — could serve as a grim warning to athletes and trainers in other countries who might feel tempted to resort to unauthorized aids. The status of the document, however, is still far from decided. Attempts by the "Smolosky" human rights group in the United States to bring it to the attention of the International Olympic Committee in Los Angeles proved unsuccessful.

Part of the explanation for that may have been the absence of a Soviet Olympic team, which in turn is held by many in Eastern Europe as well as the West to have been caused by Soviet reluctance to submit athletes to the sophisticated drug tests installed at Los Angeles.

Vera Rich

Heavy-ion research

European ambitions multiply

GANIL, the new French accelerator for heavy ions at Caen in Normandy, is to close down for January to save electricity. GANIL must save 12 per cent as its contribution to last year's budget cuts, but director Claude Detraz is not perturbed. "We're stopping in January when electricity is most expensive", he says, "and anyway we're finding we've got plenty to do when the machine's off."

In fact, Detraz is buoyant about the performance of his accelerator. He is even happy with his budget — which he believes to be quite sufficient to "sweep the field" in intermediate energy heavy-ion collisions. Detraz claims that GANIL is three years ahead of any other accelerator in performance and beam intensity and is making many interesting — and unexplained — discoveries. What GANIL needs, he says, is not money but a team of committed theorists to help explain the effects being revealed.

The accelerator at Caen is the latest of a clutch of European heavy-ion machines (others are in West Germany and Britain) dedicated to the study of the gross properties of nuclei rather than the structure of nucleons and other particles, with which high-energy physics is now preoccupied.

Detraz says he is enticing French theorists to GANIL with discoveries such as that of isolated excited states of the nucleus 100 MeV above the ground state (where previously only a continuum of states was expected), the apparent failure of thermodynamic models of the excited nucleus and strong forward scattering (a kind of nuclear transparency) at total nuclear energies above 40 GeV. Other GANIL products (detectable because of high beam intensity) are exotic neutron-rich nuclei such as nitrogen-23, neon-29 and neon-30 as well as highly-charged positron-emitting atoms. "It's a gold-mine", says Detraz.

GANIL, however, is not alone in the mine. In Britain, working another seam, the Nuclear Structure Facility (NSF) may have taken a lead in high-spin nuclear physics, where nuclei are spun to such angular momenta that Coriolis and centrifugal forces are comparable with nuclear forces. NSF is planning a major extension of its Van de Graaff accelerator, the largest in existence, by means of a superconducting linac.

In West Germany also, the heavy-ion laboratory (GSI) near Darmstadt is basking in the discovery of elements 108 and 109, and has won outline approval for a substantial extension. And Italy is building an advanced superconducting cyclotron in Milan, although the project was set back recently by the untimely death of its designer.

GANIL itself uses a pair of separated-sector cyclotrons to accelerate a wide range of nuclei from 100 MeV per nucleon (for

light ions) up to 10 MeV per nucleon (for the heaviest). In low-energy nuclear accelerators (such as NSF), nuclei approach at velocities that are small compared with the internal velocities of their nucleons, so that internal structures are rearranged before collisions. At much higher energies (such as the GeV per nucleon available at Berkeley, California), on the other hand, collision velocity is much faster than the internal motions, and nuclei collide like bags of stationary separate particles. But at GANIL — the intermediate region — the velocities are roughly equal, so that simple approximations do not apply. Hence perhaps, the lack of enthusiasm of theorists, whom Detraz claims are nevertheless being won round by the challenge of GANIL.

NSF (at Daresbury, Cheshire) is making its mark because of its stable and turnable particle beams (a consequence of electrostatic acceleration), the range of nuclei which can be accelerated ("virtually the whole periodic table" according to a Daresbury experimenter), and a superb detector of gamma-emission from high spin states which can discriminate against Compton-scattered radiation.

Daresbury now plans to abandon the original target of a 30 MV terminal voltage, to stay with the present 20 MV but to put all its effort into developing a superconducting linac stage after the Van de Graaff. This would give NSF the capability of colliding uranium with uranium. The proposal has not yet gone to the nuclear physics board of the Science and Engineering Research Council that would fund it, but the extension could be built segment by segment without increasing the budget, they say in Cheshire.

Meanwhile, GSI Darmstadt plans to add a synchrotron and a storage ring to its present UNILAC linac (which is capable of colliding nuclei at around 20 MeV per nucleon). The additions would bring GSI up to 1.3 GeV per nucleon at high intensity, making possible a search for new states of condensed nuclear matter. The GSI additions are all but finally approved, a federal government spokesman in Bonn said last week, with a decision due "before the end of the year".

With work in this field also at CERN (the European Organisation for Nuclear Physics near Geneva) and progress in Italy, the chauvinistic cry is getting louder in Europe that the old continent has taken "an unquestionable world lead" in nuclear matter studies. That, of course, was what the particle physicists were saying until President Reagan gave the go-ahead to the super-conducting super-collider now being built as an adjunct to the Stanford Linear Accelerator Center and which may steal a march on CERN's new large electron-positron storage ring.

Robert Walgate

Japanese research

Companies go for basics

Tokyo

A BUILDING boom for basic research laboratories is under way in Japan — but neither the government nor the universities can take credit. Instead it is large private corporations that have begun to invest in academic research.

The latest is Meiji Milk Products, whose brand-new Institute for Basic Research in the Life Sciences, located in Odawara City, some 80 km from Tokyo, will be completed in the next few weeks. The initial outlay has run to 5,000 million yen (\$21 million). Around 50 scientists will be employed, together with 50 support staff, to carry out research in eight divisions spanning the whole of molecular biology.

Before Meiji Milk came onto the scene, some fifteen other companies had opened basic research facilities this year alone. The biggest surge has come for the life sciences, with six new laboratories — and a demand for researchers that has sent head-hunters in search of Japanese working in US and European laboratories. Close behind come laboratories for new materials and electronics. Famous names include robot-builders Fanuc, whose factory, where robots build robots, was toured by Mrs Margaret Thatcher, the British Prime Minister, and Toshiba, with a new very large scale integrated circuit research laboratory. Almost all the new high-tech laboratory fields are represented: artificial intelligence, molecular memories and conducting polymers all have their new facilities.

At Meiji Milk's Meiji Institute of Health Science (which bears an "MIH" logo reminiscent of a certain US laboratory) there is no pressure at all to perform applied research for company products, according to Norio Nakatsuji, until recently at the MRC Mammalian Development Unit in London and now head of the new institute's developmental biology division. He also points out that recruiting highly qualified staff has proved easier than expected — some have given up tenured positions at universities to join the institute where, with new facilities (including four P2 and two P3 biological containment research laboratories) and generous budgets, there will be no limits on research.

The basic laboratory boom is a very recent phenomenon — only one such laboratory was set up in 1982 and seven in 1983 — but it seems likely to continue. Fifteen companies have already announced plans to set up basic facilities in the near future.

What exactly is the thinking behind the boom and why are corporations prepared to spend huge sums of money to set up these laboratories? Financial analysts admit to finding the trend quite puzzling. Increased prestige is one factor they all cite,

adding that prestige is often reflected in higher stock market prices when plans for basic research facilities are announced. But the real factor seems to be an underlying feeling that Japanese corporations have drawn level with or passed their Western competitors in mastering applications of current technology, and must now look for the seeds of new technology in their own basic research rather than continuing to import ideas or to look for ideas in their own universities.

Barriers between universities and industry are said to be just too high for

effective research cooperation, and the new laboratory boom has not been accompanied by a boom in either university endowments or research contracts at universities. Instead, corporations seem intent on giving select groups of their own researchers the kind of freedom that they would enjoy in universities.

Given this trend, it is likely that the difference between Japan, where industry pays two thirds of the total research and development bill, and countries like the United States, where it pays only 40 per cent, is likely to increase — and Japanese university laboratories will have finance, equipment and technical support far inferior to those of their industrial counterparts.

Alun Anderson

Virus diseases

Immunogen for herpes, AIDS?

A DRUG developed as long ago as the early 1970s is now being hailed by its makers, Newport Pharmaceuticals of California, as a possible "new hope in herpes and AIDS". Unlike the recently introduced drug acyclovir, the "new" drug (inosine pranobex, new UK trade name Immunovir) acts by stimulating the patient's immune system, not directly against the virus.

Going by the results so far, however, it is either too early or too optimistic to welcome Immunovir as a major weapon against either genital herpes or AIDS (acquired immune deficiency syndrome, linked with human T-cell leukaemia virus type III). Clinical tests, carried out by Dr R.D. Miller and co-workers at the University of California's Irvine Medical Center, have indeed shown an effect on recurrence rate in genital herpes. In 35 patients given Immunovir tablets, 18 (51 per cent) had only 0 to 1 recurrence during the six-month study, compared with 8 of 41 (19 per cent) on placebo. The relatively high apparent response to placebo, inevitable when considering symptoms that recur at unpredictable intervals, makes it impossible to assess the efficacy of Immunovir until more clinical trials have been completed.

But the initial clinical trials do suggest that Immunovir significantly reduces viral shedding — the number of viruses present in the body's secretions. This raises the hope that the spread of herpes might be controlled by treating infected individuals. The US Center for Disease Control (CDC) estimates that there are between 5 and 20 million cases in the United States, with some 300,000 new cases each year. Herpes is not a notifiable disease in the United States, hence the uncertainty of CDC estimates. Dr Miller's own estimate is as high as 30 million cases.

Whatever the true figure, there is clearly a place for a drug that would both reduce discomfort (number of recurrences) in those with the disease and reduce the possi-

bility of them passing it on. The makers of Immunovir claim that it is cheap enough (compared with extremely expensive antivirals) and safe enough to be used in such a role. No side effects have yet been found, it is claimed, although the drug is metabolized to uric acid so cannot be used in patients with gout or kidney disorders. For treating primary herpes, with the aim of a cure that would mean no further need for treatment, it seems likely that an antiviral drug such as acyclovir will be necessary.

Immunovir has not yet been used to treat patients with AIDS, although Dr Joyce Wallace and Dr J.G. Bekesi of Mount Sinai Hospital and the AIDS Medical Foundation, New York, have treated patients with "pre-AIDS". That condition is characterized by depression of the immune system and lymphadenopathy, and may or may not develop into AIDS as defined by CDC. In these patients, as in others given Immunovir, the number and activity of natural killer cells in the blood and the number of (antibody-stimulating) T-helper cells were increased.

Improvements in laboratory tests of immune function may not always be accompanied by a clinical response. But in the 16 pre-AIDS patients with significantly improved immune responses (of 20 treated), 5 reported a subjective improvement in symptoms and were well a year after the study. Clearly, as Dr Wallace stresses, it is too early to say whether Immunovir is likely to revolutionize the treatment of AIDS and pre-AIDS, although she is encouraged to carry out further trials, and a new trial is due to begin in London later this year.

Although publicity for Immunovir has centred mainly on herpes and AIDS, the drug has shown some promise in a variety of virus diseases — hepatitis, mumps, subacute sclerosing spindylitis and influenza among them. Initial results, however, seem to suggest that amelioration of symptoms is more likely than a complete cure.

Charles Wenz

Australian science

Budget dismays even ministers

Canberra

If they needed reminding of it, Australia's scientists had their political impotence brought sharply home to them when the federal budget was announced at the end of August. In a fiscal strategy aimed at promoting business confidence by reducing the budget deficit to \$A6,745 million (\$4,500 million) from last year's \$A7,961 million, and smoothing the Labor Party's path to re-election by means of a small tax cut immediately before the federal polls (now tipped for November or December), the feebleness of the science lobby was made painfully obvious.

The science and technology minister, Mr Barrie Jones, is reported to have contemplated resignation from the ministry over the budget cuts to his portfolio, and later to have earned the prime minister's displeasure by hinting publicly that taxation would increase after the election. Beyond exhorting scientists to make the public more aware of the value of their work, Mr Jones did not elaborate on the tactical details of the successful science lobbying.

Including government appropriations for salaries, administration and major capital expenditure of \$A596 million — a monetary increase of 1.6 per cent over the 1983–84 figure — funds totalling \$A660 million will be available through the Department of Science and Technology, if industry subventions and earned revenue are taken into account. Complaining of the new budget's effect on morale, Dr Paul Wild, chairman of Australia's largest research group, the 7,000-strong Commonwealth Scientific and Industrial Research Organisation (CSIRO), reported that CSIRO's direct budget appropriation of \$A322 million intends no provision for inflation in non-salary operating funds and that most areas of CSIRO research will face cuts of up to 4.3 per cent.

An estimated 180 CSIRO jobs will be cut, mostly those of young scientists in short-term non-tenured positions. Already, the staff of at least one CSIRO division have agreed to take leave without pay to bring down salary costs and minimize retrenchments. To keep things in perspective, though, the government has set aside \$A30 million for Australia's defence of the America's Cup yacht race in 1987.

The science portfolio has suffered considerably compared with the others, which is especially disappointing given the importance accorded it in the national technological strategy. The government's total outlays, excluding health, represent an average monetary increase of 10.7 per cent across the whole economy. With a projected inflation rate of 6 per cent, the 1.6 per cent monetary increase for government-sponsored science and technology

results in a net contraction of about 4.5 per cent in real terms.

At \$A1,237 million, tertiary education commission grants for universities have kept pace with inflation and grants to colleges of technical and further education have increased by 23 per cent to \$A307 million. The Australian Research Grants Scheme, however, has had its funds augmented by only \$A3.5 million to \$A26 million to meet grant applications for 1985 totalling \$A65 million.

Budget provision for medical research grants has been increased by \$A6 million to \$A44 million. Defence science has an extra \$A11 million, taking its budget to \$A158 million. Support for the Bureau of Mineral Resources has increased from \$A23 million to \$A31 million.

US election

Shoestring lobbies

Washington

In an election campaign that has seen optometrists and freight-forwarders organize Political Action Committees (PACs) to channel money to candidates favourable to their causes, scientists seem almost strangely absent. The records of the Federal Election Commission reveal only a handful of PACs concerned with scientific issues, and these, by any standard, are woefully impoverished.

Organizers of science-oriented PACs blame their poor showing on an "ivory tower" attitude among scientists who refuse to see scientific interests as the basis for political organization, and on the "bad name" that PACs have received. The result of post-Watergate election reforms, PACs are the only legal means by which organizations not affiliated with a candidate can make campaign expenditures. Although PACs are allowed to contribute no more than \$5,000 to any one candidate, there is no limit on the amount that can be spent on "independent" advertising opposing a candidate; PACs have come to be associated with such "negative advertising" campaigns.

The Science and Technology PAC, or SCITEC-PAC, which claims to represent the interests of research scientists most directly, has so far spent only \$643 during this congressional election; it has made no campaign contributions at all this year. The organization's treasurer, David Garin, says that the group plans to donate a total of \$1,000 to about a dozen candidates in the next three weeks. Garin said the average contribution to the PAC was \$40 per person. (Corporations and labour unions are not permitted to contribute to PACs.)

At least two somewhat more successful PACs concentrate on space issues. The Campaign for Space, which has the stated

An addition of \$A150 million over five years to the research funds available for automobile design through the Australian Industrial Research and Development Incentives Board arises from the design facility of the Button car plan, formulated by Industry and Commerce Minister, Senator John Button.

Mr Jones is nevertheless bravely burying his disappointment by a recitation of the bright spots in the budget. Antarctic research and the Bureau of Meteorology are to enjoy considerable increases, and modest support will be given to marine sciences, the National Biotechnology Scheme, fifth generation computing and a new CSIRO Division of Information Technology. Nonetheless, the minister's exasperation could not be disguised. He remarked that in an election year, short-term political considerations will always displace the longer perspective necessary in science.

Jeffrey Sellar

aim or promoting a balanced manned and unmanned civilian space programme, including both construction of a space station and an increased programme of planetary exploration, has taken in about \$40,000 for this election; the group plans to contribute \$10,000 to congressional and senatorial races. The group has also endorsed the re-election of President Reagan, citing his support for the space station and Democratic challenger Walter Mondale's opposition to the shuttle when he was in the Senate.

The other space group, called SPACEPAC, has taken in \$60,000 for the election, spending almost all of it so far on fund-raising costs. Its only campaign contribution has been one of \$300 to Representative Don Fuqua, chairman of the House of Representatives Science and Technology Committee.

Thomas Frieling, executive director of the Campaign for Space, readily acknowledges that the small contributions of his and other science-related PACs cannot make much difference to the election results. But, he notes, even the "big league" PACs are limited to contributing \$5,000 to election campaigns that cost hundreds of thousands of dollars in the House and millions in the Senate. Garin, of the SCITEC-PAC, suggested that the real significance of his contributions was that they represent an endorsement by a non-partisan group that has the interests of science at heart.

The one scientific professional society that has organized a PAC, the National Society of Professional Engineers, dwarfs these efforts; it has so far raised \$200,000 for this election. And nobody else comes close to the American Medical Association's \$3.6 million war-chest.

Stephen Budiansky

Peer review

SIR — The problems of peer review procedures deserve serious consideration, but the remedy proposed by M.C. Goodall (*Nature*, 23 August, p. 620), an effective appeal process, is not practicable. Grants committees have little difficulty in identifying outstanding or mediocre proposals; it is the predominant mass of attractive sound applications that cause the problems. In the "good old days", virtually all of these would have been supported. Today a large proportion fail, their fates being determined by slight variations in the (usually secret) scoring by various members of the committee. Mean scores determine a rank order which is the basis for allocating funding from the top of the order until the funds are exhausted.

It would be quite iniquitous to review an appeal a single application that had failed without also reviewing all the competing (even successful) applications, perhaps even including those that were successful. Who is to say that a second complete review would be any more just than the initial review? In a situation of diminished resources it has to be accepted (albeit reluctantly) that a considerable element of chance is an inescapable component of today's review procedures.

The only marginal improvement that might be worth considering would be a more sensitive scoring system in the critical region around the anticipated cut-off point. For example, at present one major grant award-body scores from 0 (bad) to 6 (outstanding) with a funding cut-off in the region of 3.7. In practice most good applications are scored either 3 or 4 by committee members and the mean score can be moved from one side of the cut-off point to the other by just one member altering a score from 3 to 4, or vice versa. If members could score in fractions in this region rather than integers they could exercise greater discrimination, giving a somewhat less chancy mean score.

BRYN BRIDGES

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Nature bingo?

SIR — It occurs to me that the current "Bingo War" in Fleet Street may pose a serious threat to the maintenance of *Nature's* circulation. May I therefore suggest a way in which this might be countered? I propose the institution of a new game entitled "Base Sequence". Each subscriber would be sent his own "DNA card" on which would be printed a unique sequence of 36 bases. Three or four tRNA anticodons would then be printed in each issue of *Nature* (possibly at the bottom of the contents page), the winner being the first reader to match up his base sequence with a complete set of complementary

triplets, thus completing his peptide. Obviously frame-shifts and "spare" bases could not be allowed!

I feel certain that such a course of action would assure the future of the journal, the only possible disadvantage being the risk of attracting the eye of Rupert Murdoch or Robert Maxwell.

S.J. PUBLICOVER

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Brazilian science

SIR — Your special offer for Brazilian scientists to purchase *Nature* is important not only in helping Brazilian scientists to keep themselves up to date in science, but also in calling attention to the isolation from the international scientific community of Brazilian scientists due to the present policy of budgetary restrictions for science and education. The faculty members of the Brazilian federal universities are involved in a national struggle for better working and teaching conditions. Among other facilities that are becoming inoperable, the libraries are at the moment incapable of renewing their subscriptions due to lack of money for education in addition to the restrictions on foreign exchange.

MARCELLO ANDRÉ BARCINSKI

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Bio company launch

SIR — Since *Nature* has a long standing interest in promoting advances in biotechnology, we have chosen to make the first announcement of our new venture in your pages.

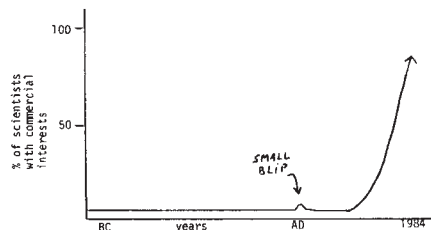
At a recent meeting of the American Society of Biological Chemists (ASBC, San Francisco, June 1983), we were amazed by the number of biochemists who are owners, co-owners or owned (as "consultants") by private enterprises. Our unbiased survey of participants at this meeting revealed the following (see figure).

At the time of Adam and Eve, less than 0.001 per cent of all living scientists were engaged in profit-yielding activities. Much later (see small blip in curve) a sizeable percentage of scientists convinced the public that lead could be transformed into gold. In the 1980s, this logic has been extremely successful^{1,2}. There are a number of explanations for the rapid increase shown in the figure other than blatant greed including (1) new techniques for producing monoclonal antibodies; (2) gene cloning; (3) gullibility of the public.

At the San Francisco ASBC meeting, we were quite embarrassed not to have an official company business card, as did all our friends. Furthermore, when walking back to our sleazy hotel (Taylor Street, *et al.*) late one night, we were approached by

two charming women asking "would you like a little company?" This encounter sparked our interest and we decided to start our own enterprise.

As indicated on our tastefully designed letterhead, Genasex was conceived. The response has been fantastic. In our travels, many scientists have expressed keen interest and overt jealousy. Suggestions



arrive daily. One colleague advised changing our name from Genasex Ltd to Genasex Unlimited. A colleague from Milan offered to head an Italian subsidiary to be called Gen-italia, but we have postponed a decision on that.

What products does our company produce? So far, none. The reasons for this policy include (1) our observation that many of the new biotechnological companies also produce no products; (2) of those who do, most are in the red; and (3) to use this company as an effective tax write-off, we must be careful not to be too successful.

Hitherto, our financial investment has been negligible. Naturally, we solicit readers' contributions and would certainly consider adding names to our board of directors. Our real hope is that another company, perhaps with a similar name, would offer to buy us in order to avoid the inevitable competition.

D. MALAMUD

J. HANOUNE

*Genasex Ltd,
c/o University of Pennsylvania,
Thomas W. Evans Museum
& Dental Institute,
4001 Spruce Street,
Philadelphia, Pennsylvania 19104, USA*

1. Dow-Jones *et al.*, any day, any paper.
2. *Nature*, Index of Biotechnology Stocks, monthly reports.

Photocopying

SIR — J.D. St Aubyn (*Nature* 304, 678; 1983) correctly stresses that it is publishers and not authors who are concerned about large-scale copying. In fact most reputable suppliers of tear sheets or photocopies, ourselves included, adhere to the requirements of the Copyright Clearance Center (CCC), so eliminating most of the administrative copying.

The price of an authorized photocopy includes the CCC payment, I believe the British Lending Library has agreed to pay CCC fees on all photocopies mailed to the United States.

EUGENE GARFIELD

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Jumping the smoking gun

Are antibiotics in animal feed a threat to human health? Contrary to some opinions, the latest evidence put forward in the United States is inconclusive.

ALTHOUGH Western Europe has now banned the use of antibiotics as a growth-promoting additive in animal feeds, the United States has since 1977 — when a proposed ban was blocked by Congress — maintained that additional data are needed to justify such an action. Half of the 35 million tons of antibiotics manufactured each year in the United States end up in animal feed; not surprisingly, the prospect of a ban is not looked upon favourably by the pharmaceutical industry.

But neither is it by farmers. The evidence, as paradoxical now as it was when the growth effect was discovered in 1949, is nonetheless convincing that chickens, pigs and cattle do much better when fed subtherapeutic doses of antibiotics. Even when the animals show a significant percentage of resistant bacteria in the gut, the effect persists; chickens, for example, will gain weight as much as 10 per cent faster when fed antibiotics¹.

Balanced against this convincing economic benefit, however, is mounting concern about the spread of resistant organisms. The discovery that resistance can be conferred by mobile plasmids, and the appearance of certain resistant strains of gonorrhoea, *Haemophilus influenzae* and dysentery-causing organisms, has led to repeated calls for cutting back "inappropriate" use of antibiotics; a prime target is their use in animal feeds. The case has always been circumstantial: although it is known that resistance-conferring plasmids do occur in animal-borne microorganisms and that plasmids can readily be transmitted to other bacteria, there has been a lack of convincing proof that the end result actually is the production of resistant strains of human pathogens. A major complication in any effort to establish such a link is the other half of the US antibiotic production, the half that ends up in humans, not infrequently for reasons that range from the inappropriate (ingrown toenails) to the unethical (as placebos for treating colds).

Now, proponents of a ban claim to have found the smoking gun. A study published last month in the *New England Journal of Medicine*² traces an ampicillin-carbenicillin-tetracycline-resistant salmonella infection in 18 persons to a beef cattle herd that was the probable source of hamburger eaten by at least 13 of the patients. The cattle had been fed subtherapeutic amounts of chlortetracycline; no therapeutic doses had been administered. The

bacteria in all of the patients showed identical resistance and contained a 38-kilobase R plasmid; and these bacteria were identical with salmonella isolated from the tissue of a dairy calf that died of scours on a farm adjacent to the beef feedlot. No samples of hamburger were tested, however. In an editorial in the same issue of *NEJM*³, Stuart Levy of Tufts University, a longtime critic of the use of antibiotics in animal feed, asserts that this study establishes an "important missing link", and concludes that "the clarion is strong and clear".

But the clarion may sound differently, depending on where one is sitting. Salmonella is one of the few pathogens common to animals and humans; it is facile to point to this case as proof that subtherapeutic antibiotics in animals can lead to antibiotic-resistant diseases in humans. Salmonella is a dangerous organism whether it is resistant or not. It should never be present in meat, whether it is resistant or not. Treatment of salmonellosis does not involve the use of antibiotics. The entire story makes a stronger case for ensuring proper sanitation in meat processing and for cooking your hamburger than it does for banning antibiotics in cattle feed.

Even more to the point, 12 of the 18 patients had been taking antibiotics, most for questionable reasons, when they became ill. Three had taken the drugs without the direction of a physician, using pills left over from an earlier prescription or from a family-member's prescription; the others had had them prescribed for sore throats or bronchitis. Because the gastrointestinal symptoms appeared very rapidly after the patients began taking the drugs (usually a matter of 1–2 days), it seems likely that they already had an asymptomatic infection that flourished as soon as competing, non-resistant bacteria were wiped out by the antibiotics. The important conclusion that cannot be overlooked is that if those concerned had not been taking the drugs — and most of them probably should not — they would not have become ill. (Much is being made of the fact that one of the 18 patients died. According to the authors, he received his infection from an incompletely disinfected sigmoidoscope that had just been used on one of the salmonellosis patients.)

Rather than a "compelling" case for the banning of antibiotics from animal feed, as Levy would have it, the study begs the

question whether animals are a significant generator of resistant human pathogens. Just as it has been insufficient merely to lay out the theoretical possibility that resistance in animal bacteria could be transferred to human pathogens (a possibility that no one disputes), so it is insufficient to demonstrate, however elegantly, that meat contaminated with resistant salmonella can make people ill. The real question is the extent to which resistance engendered in animal bacteria is responsible for the spread of resistance to strictly human pathogens, such as gonorrhoea. Levy's argument that we have to worry more about antibiotics fed to animals than antibiotics fed to humans because animals produce more faeces is unconvincing. The real missing link is the extent to which human pathogens have an opportunity to interact with animal pathogens and to acquire resistance plasmids. This is surely the limiting factor in determining the animal-feed contribution to resistant human pathogens. Speaking vaguely about the "environmental pool" of resistance plasmids makes little sense in the absence of an answer to this question.

One recent attempt to look at the big picture in fact suggests that there are mechanisms in the environment that indeed limit the spread of resistance. Barbara Atkinson and Victor Lorian have reviewed tests for resistance made on 43,250,000 bacterial samples in 242 hospitals from 1971 to 1982 and have found no overall increase in resistance to 15 different antibiotics during this period⁴.

Indeed, the very fact that none of the bacteria residing in the gut of farm animals is resistant suggests that some equilibrium has been reached. If, in a couple of hundred thousand generations, bacteria have not become totally resistant in the face of assault by subtherapeutic antibiotics, is it merely complacency to assume they never will? It seems plausible that the energy cost to the bacteria of maintaining resistance plasmids is at least one counterbalancing force in the equilibrium equation.

If nothing else, all this should convince us that we do not risk imminent disaster by delaying regulatory action until we understand better the spread of resistance plasmids.

Stephen Budiansky

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Materials science

Making fuel for inertially confined fusion reactors

from Robert W. Cahn

THE proceedings¹ have recently been published of the Third Topical Meeting of Fusion Reactor Materials, held in September 1983. The very varied and exhaustive papers on tokamaks and their numerous materials problems include nothing whatever on the design and fabrication of the fuel micropellets needed for inertially confined fusion (ICF) reactors. Indeed, ICF is not mentioned anywhere. What accounts for this absence?

The previous meeting in this biennial series (held in August 1981)², and a brief attempt to gaze into the future of fusion power, published in the same year³, likewise had no mention of this important topic. If, however, we go back to the First Topical Meeting⁴, held in January 1979, we find a group of nine papers concerned with ICF fuels, covering in considerable detail the position at that time concerning the design of the fuel microspheres, their fabrication and characterization, and the tricky problem of protecting the first wall of an ICF reactor from rapid series of mini-explosions. Why the near-total silence since then?

A very readable review paper on 'the special materials in laser fusion targets' has just been published⁵. The manuscript was received by the editor of *Res Mechanica* in April 1983, and the author states that he is presenting "the state of the technologies in making these materials, and problems encountered in their development, as of March 1980". So it seems, possibly because of the connection of these technologies and problems with the military uses of lasers, that a security blackout descended after March 1980. I have seen a reference to a "1980 Topical Conference on Inertial Confinement Fusion in San Diego" but no indication that the proceedings were published. Even with what we have, however — the 1979 papers and this 'postdated' 1980/1984 paper — it is possible to see the quite extraordinary complexity of the problems and techniques involved. Hendrick's⁶ assertion that "pellet fabrication is not a trivial task as implied by some reports; it is also not the impossible task implied by others" is to be noted.

ICF reactors are designed on the basis of the inertial confinement principle — a huge energy pulse imparted by laser or particle beam implodes a spherical shell of frozen deuterium-tritium mixture with a view to satisfying the Lawson criterion and setting off a series of microthermonuclear fusion explosions, the heat from which is absorbed and utilized to make power. In their review paper Farnum and Fries⁵ discuss the early stages of the development

of a complex laser fusion target designed for maximum fusion yield when irradiated with 100 kJ of infrared light from a CO₂ laser with 1 ns pulses. The essential parts consist of a glass microballoon coated internally with a layer of deuterium-tritium fuel and externally with a dense 'pusher' layer, the whole separated by plastic foam from an external dense metal pusher layer which is itself coated by an ablative plastic layer which creates the 'rocket' effect.

The difficulties arise from the extreme precision needed in the sphericity, thickness uniformity of layers, smoothness of surfaces (to within a few nm) and uniformity of density, for instance of the plastic foam — and all this for microspheres usually well below 1 mm in external diameter. Microballoons can be bought commercially at \$10⁻⁹ each, but their quality is hopelessly inadequate; the new paper outlines methods, starting either from liquid drops or from solid fragments in free fall, for making balloons with the requisite geometrical perfection. These thin shells can be filled with a gas mixture while in the gel state and then vitrified. (The method for turning the gas filling into a liquid spherical shell that is then frozen so rapidly that gravity has no time to distort it was described earlier⁷; this is really a kind of splat-quenching direct from the gas phase.) Problems connected with the buckling of the microballoons under gas pressure, and the effect of thin coatings on the buckling resistance, are discussed by Farnum and Fries.

The second part of their paper deals comprehensively with ways of making smooth uniform coatings of metals, polymers or a combination of these, by various forms of physical or chemical vapour deposition. These methods often depend for their efficacy on methods of levitating the spheres to be coated, either by systems of gas jets or even by whirling them around, during coating, in a form of synchrotron⁸. Remarkably perfect polymer coatings can now be produced; one method is to build up monolayers of 100 nm-diameter latex spheres under electrostatic attraction, discharge the layer and start again. Production of polymeric foam to the requisite specification, however, is still not possible to the necessary uniformity (or at least was not in 1980).

One of the rare publications to appear since 1980 that deal with ICF pellet (or 'target') production is a paper from Livermore National Laboratory describing the use of an array of gas microjets to levitate a pellet, to allow an ultraspherical polymer

surface layer to be made by plasma polymerization⁸.

One curious technique that is not discussed in Farnum and Fries's paper is the approach to making thin flanged micro-hemispherical shells by methods based on microelectronic technology. For instance, starting from circular etch-windows in a silicon surface, hemispherical depressions of high perfection can be etched; these are then boron-doped by diffusion into a thin surface layer and etched from the back with an etchant that does not attack boron-rich silicon⁹. It is a striking tribute to the micromechanical precision of modern silicon handling that the necessary smoothness and sphericity can be attained in this manner.

Others have recently urged the use of metallic glass microballoons and this is exciting much interest^{10,11}. Such hollow microspheres can be formed by exploiting the instabilities of hollow liquid jets. Microballoons of a dense Au-Pb-Sb glass have been made with surface smoothness and sphericity of a very high order. How such balloons are to be incorporated in a complete target is not disclosed. Such metallic glasses are known to be highly permeable to hydrogen and thus should not be difficult to fill with fuel. More generally, Lee has very recently outlined a range of other uses for metallic microballoons made from unstable hollow jets, including a rocket fuel¹². A new technology seems to be emerging.

It would seem that a new generic name is required for the congeries of techniques which have been developed to make ICF fuel pellets — 'materials microtechnology' perhaps? When the full account of this work is published, it will be seen as an essential part of the history of modern materials science and technology. But we shall have to await wholesale declassification before we can know how successful the ICF approach to nuclear fusion currently is. Meanwhile, the interested reader is referred to a concise textbook¹³ on thermonuclear fusion that discusses the essential characteristics of inertial confinement. □

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Geology

Seismic reflections of the continental crust

from Simon L. Klemperer

A recent symposium* provided the opportunity for scientists from seventeen countries to discuss their most recent results on the structure of the deep continental crust using reflection seismology. Unusually for a technique used in pure science, reflection profiling was originally developed commercially for exploration of sedimentary basins, and therefore is available for immediate application without prohibitive developmental expense. The meeting demonstrated its versatility as a means to investigate a wide variety of geological issues in the crystalline rocks of the crust.

Many of the most dramatic successes of reflection seismology have come through tracing fault-plane reflections to surface outcrop. This has resulted in the discovery of major crustal features such as the Southern Appalachian overthrust, and the Outer Isles thrust. When basement reflections cannot be tied to information from surface exposures or boreholes, their interpretation may be ambiguous. The use of forward modelling, that is, estimating the expected reflection character from basement features observed at the surface, as a first step towards interpretation was demonstrated by J. Percival (Geological Survey of Canada) for the Kapuskasing granulite zone and the adjacent amphibolite-grade Wawa gneiss belt. He speculated that the irregular horizontal-to-discordant intrusive boundary between these metamorphic zones might appear as a Conrad discontinuity in a refraction experiment. Felsic-mafic layering in the granulite zone might be the cause of layered reflections in the lower crust, contrasting with the largely felsic (reflection-free?) gneiss belt above the granulites.

The origin of highly-reflective layered lower crust is obscure, and has attracted considerable speculation. Two contrasting theories were proposed with several workers arguing for magmatic processes, such as underplating of the crust, as the primary cause for the reflective layering with extensional shear or thrusting taking a secondary role.

By contrast D. Matthews (Cambridge University) proposed the existence of lamellae of hydration or of high pore-water pressure as the major cause of lower crustal reflectivity. The presence of water or graphite in the lower crust would reconcile the observed highly-conductive zones in the lower crust with data from laboratory experiments. Clearly, more detailed geo-

physical and geochemical modelling as well as more detailed geological mapping are called for.

Clear reflections at about Moho depth, or bands of reflections terminating at about Moho depth, are not uncommon on deep reflection profiles, particularly extensional terranes, as seen, for example, in the Consortium for Continental Reflection Profiling (COCORP) data from the Basin and Range, and in the British Institutes Reflection Profiling Syndicate (BIRPS) data from the British marginal seas. Near-vertical reflection seismology does not accurately define velocities at Moho depths, but it is in terms of a velocity change that the Moho is conventionally defined. In the absence of velocity data, the reflection Moho must be picked on the basis of seismic character.

P. Barton (Cambridge University) made use of the best available set of coincident refraction and near-vertical reflection profiles to show that in the North Sea, the reflection Moho, defined as the base to the reflective lower crust, coincides with the refraction Moho. By contrast, D. Okaya (Stanford University) asserted that in the Basin and Range, the base of the layered lower crust, or the reflection Moho, is not coincident with the refraction Moho, although in this case, the available reflection and refraction profiles are in different locations. A. Hirn (Institut de Physique du Globe), using wide-angle reflection and refraction data, and D. Matthews, using near-vertical reflection data, demonstrated the existence of Moho offsets along faults, causing sudden changes in Moho depth of several kilometers across narrow zones.

The reflection character of the Moho, where observed as discrete reflections, varies from complex layering to simple single reflections (L. Braile, Purdue University; R. Phinney, Princeton University). Braile suggested that the disparity between wide-angle reflections, on which the Moho may appear as a continuous boundary, and the near-vertical reflection Moho, which typically lacks horizontal continuity, could be due to fine-scale lamination of the Moho. D. Blundell (Chelsea College) used simple 3-D ray-tracing models to show how a single surface, suitably cross-folded into an egg-box structure, appears as a thick band of reflections on a stacked seismic section, due to interference of reflected arrivals from different directions. These complex reflection patterns show the need for 3-D surveys to constrain the direction from which reflected energy is returning.

Although most reflection profiling results so far have come from the crust, which is highly reflective relative to the mantle, several speakers discussed sub-crustal reflectors in the mantle. The most dramatic mantle structure imaged by near-vertical reflection seismology so far is the Flannan thrust, a Moho-offsetting fault north-west of Scotland, known to penetrate to at least 45 km, or 15 km below the Moho (D. Matthews). An experiment (DRUM: Deep Reflections from the Upper Mantle) to be conducted later this year, using long recording times, will have the potential to trace this structure, and to search for others, down to 180 km depth.

Sub-crustal structural complexity is also suggested by wide-angle reflections and refractions showing Moho offsets, or Moho doubling, beneath some Tibetan sutures and beneath the North Pyrenean fault (A. Hirn).

Many of the data shown were collected in programs for hydrocarbon and mineral exploration, nuclear waste disposal and earthquake fault hazard reduction, as well as for academic research. J. Oliver (Cornell University) described a plan for worldwide cooperation in undertaking a comprehensive reconnaissance of the earth's continental crust which might collect 200,000 km of deep seismic reflection profiles in a 20 year period. Although sounding impressive, this is in total only one-eighth of the mileage collected by the petroleum industry in a single year. In the United States, COCORP, now in its tenth year, has acquired nearly 6,000 km of crustal reflection data throughout the continent and there are more recent programmes including those of the United States Geological Survey (discussed by R. Hamilton), Virginia Polytechnic Institute (J. Costain), University of Wyoming (S. Smithson) and CALCRUST (P. Malin, University of California, Santa Barbara).

Programmes outside the United States include the Canadian LITHOPROBE (A. Green, Department of Energy, Mines and Resources) on the Quebec Appalachians and Vancouver Island, British Columbia; BIRPS (D. Matthews) which has recorded over 3,000 km of marine data circumscribing Great Britain; the Deutsches Kontinentales Reflexions Program of Germany (DEKORP; R. Meissner, Kiel Institut für Geophysik); the French Etude Continentale et Océanique par Réflexion et Réfraction Sismique (ECORS; C. Bois, Institut Français du Pétrole); the Australian continental reflection programme (F. Moss, Bureau of Mineral Resources) and others in New Zealand, China, India, Hungary, Czechoslovakia, Switzerland, Italy and Antarctica. Many of these groups have plans to expand their programmes considerably, and thus the global initiative outlined by Oliver should be attainable. □

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*Deep structure of the continental crust', 26-28 June, Cornell University, co-ordinated by Dr Muawia Barazangi.

Gene expression

Regulation by anti-sense RNA

from Andrew Travers

THE ability of two complementary strands of RNA to form a stable duplex offers a potentially powerful mode of regulating the biological function of RNA molecules. Thus the normal RNA transcript of a gene could be rendered inactive were it to form a duplex with an 'anti-sense' RNA transcript from part of the same DNA. The past year has produced some good examples of how bacteria employ such a form of control; moreover, the experimental manipulation of gene expression by double-stranded RNA in eukaryotic cells has recently been described.

In prokaryotes, a classic example of this type of control is the regulation of DNA replication of the plasmid ColE1. In this case, a short anti-sense transcript is believed to form a RNA-RNA duplex with the 5' end of the replication primer and as a consequence to inhibit replication (Tomizawa, J. & Itoh, T. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1981; Lacatena, R.M. & Cesareni, G. *Nature* **294**, 623; 1981). Mutations affecting either the structure or the amount of anti-sense RNA affect both copy number and plasmid incompatibility.

One example in the control of bacterial translation is the regulation of Tn10 transposase production (Simons, R.W. & Kleckner, N. *Cell* **34**, 683; 1983). The discovery stemmed from the observation that the presence of a multicopy plasmid containing the insertion element IS10 inhibits *in trans* transposition of a single-copy chromosomal Tn10 element. IS10 is itself essential for Tn10 activity, providing the functions necessary for Tn10 transposition. Simons and Kleckner found that they could delete all but 75 base pairs (bp) of the transposase-coding region of IS10 and still retain multicopy inhibition, showing that the phenomenon does not require intact transposase protein. Intriguingly, the portion of the transposase gene required contains a promoter which directs transcription in the opposite direction to the transposase gene, producing a RNA molecule which overlaps and is complementary to 36 bases at the 5' end of transposase mRNA. To determine the target of inhibition, Simons and Kleckner constructed two sets of IS10-*lacZ* fusions; in one set, the amino (5')-terminal portion of the transposase gene with its cognate promoter was fused in frame to an appropriately engineered β -galactosidase gene; in the second set, an intact *lacZ* gene was fused to transposase promoter. Multicopy IS10-containing plasmids reduced expression of the fusion protein by more than 10-fold but that of the intact *lacZ* gene by less than 2-fold. Assuming that transcription originated from the trans-

posase promoter in both cases, this result strongly suggests that regulation of transposase expression is exerted primarily by modulating translation and not transcription.

A similar situation has been described for the natural regulation of the production of the outer membrane protein encoded by the *ompF* gene (Mizuno, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 1966; 1984). This protein is one of two major outer membrane proteins of *Escherichia coli* that serve as diffusion pores for small hydrophilic molecules. The total amount of these two proteins, OmpF and OmpC, remains constant but the proportion of the two varies, depending on the osmolarity of the medium. While characterizing the promoter of the *ompC* gene, Mizuno and his colleagues observed that a DNA fragment upstream of the promoter inhibited the production of OmpF protein when *ompF*⁺ cells were transformed with a multicopy plasmid containing the fragment. They discovered that this DNA fragment encoded a small 174 base RNA, termed mic RNA (mRNA-interfering complementary RNA), which was transcribed in the opposite direction to the *ompC* gene. This RNA contains no open reading frames preceded by a ribosome-binding site and so is unlikely to encode a protein. It does, however, have extensive homology with the 5' end of *ompF* mRNA, suggesting that the small RNA could form a stable hybrid with *ompF* mRNA. Since the region of homology includes the ribosome-binding site for *ompF* translation the obvious possibility is, again, that hybrid formation between the two RNA species inhibits translation initiation. A further characteristic of potential biological significance is the coordinate regulation of the *ompC* and mic

RNA species which could provide an efficient mechanism for maintaining a constant total amount of OmpF and OmpC proteins.

For neither IS10 transposase nor *ompF* protein production has the presence of a RNA-RNA duplex been directly demonstrated. Nevertheless, the fact that anti-sense RNA molecules can inhibit gene expression has been confirmed by engineering the production of such molecules for other genes. Coleman *et al.* (*Cell* **37**, 429; 1984) constructed plasmids that produce small RNA molecules complementary to the 5' ends of the mRNA either for *E. coli* lipoprotein or for two other outer membrane proteins, OmpA and OmpC. In all cases expression of these anti-sense RNA species resulted in a substantial inhibition of expression of the target mRNA species. Significantly, anti-sense lipoprotein RNA also reduced expression of *ompC* mRNA and vice versa, a phenomenon consistent with the extensive homology between the 5' regions of the two mRNA species.

The ability of anti-sense RNA to inhibit gene expression has been shown to be applicable to eukaryotes. Izant and Weintraub (*Cell* **36**, 1007; 1984) constructed a plasmid so that a promoter directed the transcription of a RNA complementary to the normal thymidine kinase (TK) transcript. When such plasmids together with a plasmid containing a normally expressed TK gene were injected into mutant mouse L cells that lacked TK, it was observed that the presence of the 'complementary' gene substantially reduced expression from the normal plasmid.

The extent to which this novel form of regulation of gene expression is utilized naturally in prokaryotes and eukaryotes remains to be established. Clearly, however, the potential for manipulating gene expression artificially by this mechanism is substantial and well worth exploiting. □

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Sedimentology

Control of dolomite formation

from M. Kastner

A PAPER on page 450 of this issue of *Nature* by Guanatilaka *et al.* describes a recent dolomite [CaMg(CO₃)₂] formation in the subtidal zone of a shallow hypersaline lagoon in southern Kuwait¹. The finding is of interest not only because it provides further insight into the mechanism of dolomite formation, but also because it has economic implications, dolomite being an important hydrocarbon reservoir, a host-rock for base-metals and associated with evaporites.

Dolomite, one of the three most com-

mon sedimentary carbonate minerals, has long perplexed sedimentologists, for two reasons. The first is that there are marked irregularities in its distribution with time. Thus, dolomite is a common, extensive and widespread rock-forming mineral in ancient sediments, in particular in late Precambrian and Palaeozoic times, but it is rather scarce in the Holocene. Between the Jurassic and Cretaceous the ratio of dolomite (sedimentary rock composed essentially of dolomite) to limestone decreases rapidly and strikingly. Early

explanations put forward for the scarcity of Holocene dolomitites were changes in the nature of plate tectonics, correlation with extensive shelf seas, and changes in the magnesium concentration in seawater (for example, refs 2–4), but none has proved satisfactory. The second puzzling aspect of dolomite distribution is that it seems to violate the laws of thermodynamics. Dolomite is the thermodynamically stable carbonate mineral in seawater; why, then, is it relatively scarce in the widespread Holocene marine carbonate sediments?

In the late 1950s and early 1960s several small deposits of Holocene penecon-temporaneous dolomitites were discovered in marine marginal, mostly supratidal, environments. Their location suggested that dolomite formation requires a molar Mg^{2+}/Ca^{2+} ratio higher than that of seawater (5.3). This theory dominated the literature until five years ago, since when dolomite has been discovered as an important constituent of many modern marine organic-rich sediments, for example, in the Gulf of California, the California borderlands, the shelf off Peru, the Japan trench and the Cariaco Basin off the coast of Venezuela. In the Gulf of California, dolomite forms actively from marine pore fluids with a molar Mg^{2+}/Ca^{2+} ratio of 3.0 (see, for example, ref. 5). Various new models of dolomitization have been proposed^{6,8}.

Recent experimental work^{9,10} shows that the essential condition for dolomite formation is not a high Mg^{2+}/Ca^{2+} ratio, but a low concentration of sulphate ions. Sulphate ions are a major inhibitor of dolomitization of calcite and, to a significantly lesser extent, of aragonite. Mg^{2+}/Ca^{2+} ratios greater than about 0.7 favour dolomitization, but are not thermodynamically required at surface to near-surface temperatures and pressures. Marine sites conducive to dolomite formation are therefore those where dissolved sulphate concentrations are low. The most effective processes of sulphate removal from, or its dilution in, marine pore fluids are microbial sulphate reduction in organic-rich sediments and mixing of seawater with large volumes of freshwater. The high (28 millimoles) dissolved sulphate content of seawater also explains the scarcity of dolomite in thermodynamically highly-favourable environments, for example, in open marine carbonates. Other low-sulphate environments favouring the transformation of $CaCO_3$ into dolomite are $CaCO_3$ -rich sediments or rocks exposed on land or within supratidal zones through which freshwater or a mixture of fresh- and seawater circulates, and chalks and limestones through which sulphidic hydrothermal mineralization solutions circulate.

The paper by Gunatilaka *et al.*¹ is an important new study of Recent dolomite formation. On the basis of petrographical studies and chemical analyses of pore fluids, the authors suggest that dolomit-

ization of micritic aragonite is promoted by a combination of partial bacterial reduction of sulphate dissolved in pore fluid and occasional near-schizohaline conditions through seasonal rainfall.

What is still puzzling is the paucity of dolomitites formed during the last 100–200 million years relative to their abundance in older sediments. It might be related to changes in the depositional environments of calcareous sediments, and hence to an increase of the calcite/aragonite ratio of the original calcareous sediments following the appearance of coccolithophoroids and foraminifers. On the other hand, the apparent abundance of Archaean dolomitites might indicate that the content of sulphate dissolved in the early oceans was relatively low. □

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Astronomy

Meteorites from Mars

from David W. Hughes

MUSEUMS around the world have about 2,500 meteorites in their collections. Where have they all come from? Various origins have been proposed, including the Moon, Mars and asteroids. In a recent paper, G.W. Wetherill of the Carnegie Institution of Washington examines the transport of meteoritic material from Mars to Earth.

Meteorites originate as parts of much larger parent bodies, which formed by a process of condensation and accretion, mixing, depletion, melting and loss of volatiles in the primordial Solar System nebula. The process was completed very quickly, about 4.6×10^9 years ago. Over the lifetime of the Solar System, the parent bodies have collided, producing fragments which are the meteorite precursors. The diversity of meteorite types suggests they are derived from about fifty parents which themselves differed in composition as a result of the different temperatures and pressures in the various regions of the primordial nebula. Once freed from their parents, the meteorites were exposed to cosmic-ray bombardment, which produced tracks of radiation-damaged material. Analysis of these tracks indicates that the parent-body collisions occurred typically 50 million years ago for the stony meteorites and 1,000 million years ago for the iron meteorites.

As their exposure ages are so short, some meteorite parents should still exist and be an observable class of celestial objects. Possible candidates are the Apollo and Amor asteroids. Alternatively, meteorites may be the debris of disintegrating cometary nuclei or they may have been ejected from Mars, Mercury and the Moon — the major negligible-atmosphere members of the inner Solar System.

Öpik predicted that only 0.2 per cent of meteorites were of lunar origin and suggested that even fewer would come

from Mars (*Adv. Astr. Astrophys.* **4**, 301; 1966). Interest in the possibility of a martian origin for meteorites has recently been renewed, however, on account of three meteorite classes, the shergottites, chassignites and nakhlites. Their isotopic composition indicates that they crystallized as recently as $0.18\text{--}1.3 \times 10^9$ years ago. Even more unusual, they have a geochemical and petrological affinity to the rocks of the Earth's mantle. One shergottite, Elephant Morain, has trapped rare gases and nitrogen resembling the composition of the martian atmosphere.

Impact craters are being produced in the surfaces of all inner Solar System planets, including Mars. Crater-forming projectiles hit the planet at the rate of about 10^9 yr^{-1} . This produces about 10^{11} g of ejecta, most of which is melted, vaporized or pulverized and returns to the planetary surface.

In his recent paper (*Meteoritics* **19**, 1; 1984), Wetherill considers two models for the transport of meteoritic material from Mars to Earth. In the large-body one, fragments larger than 15 m in diameter were ejected from Mars about 180 Myr ago, the shock metamorphism accompanying this ejection setting the isotopic dating clock. These bodies were then broken up by collisions with small asteroids and the fragments exposed to cosmic rays. The fragments subsequently hit Earth. In the small-body model, rocks smaller than 1 m are ejected from Mars and exposed to cosmic rays immediately after ejection. The major difference between the two models is the percentage of material that can be accelerated to greater than the martian escape velocity. Calculations show that only 0.03 per cent survives in the small-body case whereas a more unrealistic 0.4 per cent is predicted in the large-body model. Theoretical work on crater formation also indicates that only small bodies

and those lying very near the planetary surface will survive unaltered by the ejection process.

The orbits of the ejected bodies have relatively low eccentricities and inclinations and can exist, on average, between 5×10^6 and 100×10^6 years (depending on aphelion distance) before being hit by another minor body in the Solar System and broken up. Calculations show that of all the material that will hit the Earth from a specific martian event, 36 per cent will do so within 10^7 years. In the favoured small-body model, Wetherill suggests that the cosmic-ray exposure ages of the meteorites are equivalent to the transit time between Mars and Earth. The much longer isotopic age represents the time interval between the present day and the time of production of the specific region of Mars' surface that suffered the impact.

Similar calculations were made for Mercury ejector and it was found that 100 times fewer Mercury meteorites would finally hit the Earth.

What remains to be explained is the ratio between martian and lunar meteorites. The shergottite-nakhlite-chassignite class of meteorites represent 8 of the 2,000 distinct recovered stony meteorites. Only one certain lunar meteorite has been found, a 31 g rock found in the Allan Hills region of

Antarctica and known as ALHA 81005. (There are two other 'possibles', both found by the Japanese near the Yamato Mountains in Antarctica.) Other things being equal, theory predicts that the influx of lunar meteorites will be at least several hundred times greater than that from Mars. By contrast, observations indicate that it is smaller by a factor of around 3. A possible explanation is that the orbital dynamics mitigates against lunar meteorites. More unlikely is the suggestion that it is due to a statistical quirk, there having been no large lunar cratering events for the last 10^5 years or more. Another possibility is that there is some special characteristic of the martian surface, the presence of volatiles for example, that facilitates the acceleration of impact-generated fragments to very high velocity.

Whatever the outcome, the importance of meteorites is obvious. The museums of the world contain about 200 tonnes of meteoritic material, of which 90 g are thought to come from the Moon, 70 kg from Mars and the rest from asteroids. Unfortunately, it seems that the only rigorous way of checking the martian origin is to go there and see. □

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Outside the temperate zone of the Northern Hemisphere, relatively little advantage has been taken so far of the opportunities offered by small sites and mor humus layers. Recently, however, Macphail has done some important work in the wet sclerophyll forests of Tasmania. These forests are dominated by *Eucalyptus* species, often reaching heights of 30–40 m, with a high (20 m) shrub layer, consisting of many species, beneath the canopy. They are found in eastern and south western Australia. Their history is uncertain because of their apparent dependence on fire as a containing factor in succession: in the absence of fire, they may be subject to invasion by temperate rain forest trees. This dependence suggests that they may be a product of human activity and maintained by it.

Macphail has now approached this question by analysing the sediments of a small (8×18 m) kettle hole in Tasmanian wet *Eucalyptus* woodland¹¹. The sediments have been buried by a landslide and date from the early Holocene (9,000 to 7,000 BP). The changes in the pollen stratigraphy of the site are associated mainly with a sequence of understorey species which can in part be explained in terms of climatic trends. Superimposed, however, is a pattern induced by fires, as indicated by charcoal frequency. A marked increase in fire frequency at about 7,200 BP is associated with Aboriginal activity in the area and this produced a distinct shift in the woodland composition in which the rain forest species, such as *Nothofagus*, which had previously been expanding, were set into decline. The involvement of fire and of man in the persistence of the wet sclerophyll forest is thus confirmed.

One further line of evidence is desirable if such studies are to be exploited to the full, namely the study of modern pollen rain in present-day forest types. In this way the varying properties of pollen production and dispersion in the trees and shrubs of the forest can be compensated for in the interpretation of fossil pollen spectra. Studies of this type in the European forests by Andersen¹² and Bradshaw² have proved very informative and one must look forward to the outcome of similar work on small sites in other parts of the world. □

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Ecology

Forest history from pollen

from Peter D. Moore

POLLEN analysis has proved a useful tool for documenting vegetational changes over thousands of years. Carried out on a regional scale, with information collected from sites, such as large lakes and mires that have been carefully selected such that local vegetational events have little impact and local noise is minimal, the technique has supplied important information on climatic history¹. To the ecologist, however, it is often the very local events that are of interest. Thus he will tend to choose small ponds, peat deposits and even organic humus layers accumulating on forest floors which receive their pollen input from the local vegetation canopy and in which regional changes in vegetation may be masked by the local flora. Such sites can provide a valuable record of local vegetational history and have recently been exploited for this purpose in many parts of the world.

Bradshaw has shown that in a forest, most pollen does not travel more than 20–30 m (ref. 2), and recently, with the help of Jacobson, he has constructed a model relating the origin of pollen components to the diameter of the receptor site³. In a small 50 m diameter site, for example, 60–70 per cent of the pollen input is immediately local in origin.

The use of mor humus layers was

pioneered in Denmark by Iversen^{4,5}, who found it a particularly useful technique for the detection of the impact of man on woodlands. His conclusion that *Ilex aquifolium*, holly, had been greatly favoured by man's modification of the Danish forests received further support from work on mor humus layers in the Netherlands by Stockmar⁶. This type of deposit has also proved useful for tracing the history of species that have limited powers of pollen dispersion and that are poorly represented in larger sites. Birks has shown that lime, *Tilia*, a tree which is known to have very local pollen deposition⁷, has been abundant in Cumbrian limestone woodland in north-west Britain from about 6,000 years ago⁸, while in southern England⁹ and in Denmark¹⁰, it declined following man's modification of the forests in AD 600–800 and AD 1650, respectively.

Erratum

IN the course of editing the News and Views article by Malcolm Walter on 'Biased record of early life', (*Nature* **309**, 512; 1984), an incorrect reference was introduced. The correct citation for ref. 1 should have been to Hofmann. H.J. *J. Paleontol.* **50**, 1040 (1976) and not to Schopf, J.W. (ed.) *Earth's Earliest Biosphere: Its Origin and Evolution*, 543 (Princeton University Press, 1983).

Geophysics

Aurora seen in daylight

from Alan Johnstone

THE long-standing question of whether aurora occurs during the day as well as the night has just been answered in a most convincing way. The answer has actually been clear from indirect evidence for many years, because the electrons, which cause the aurora when they cascade into the upper atmosphere, produce many other detectable effects as they excite atmospheric molecules to emit their characteristic light. In fact, aurora occurs all round the magnetic pole in an oval offset towards the nightside. It is there during the summer days of midnight sun as well as during the long weeks of darkness in winter. Sunlight seems to have no influence on the occurrence of aurora. The difficulty until now has been that none of the methods used to detect aurora gives a picture of its structure.

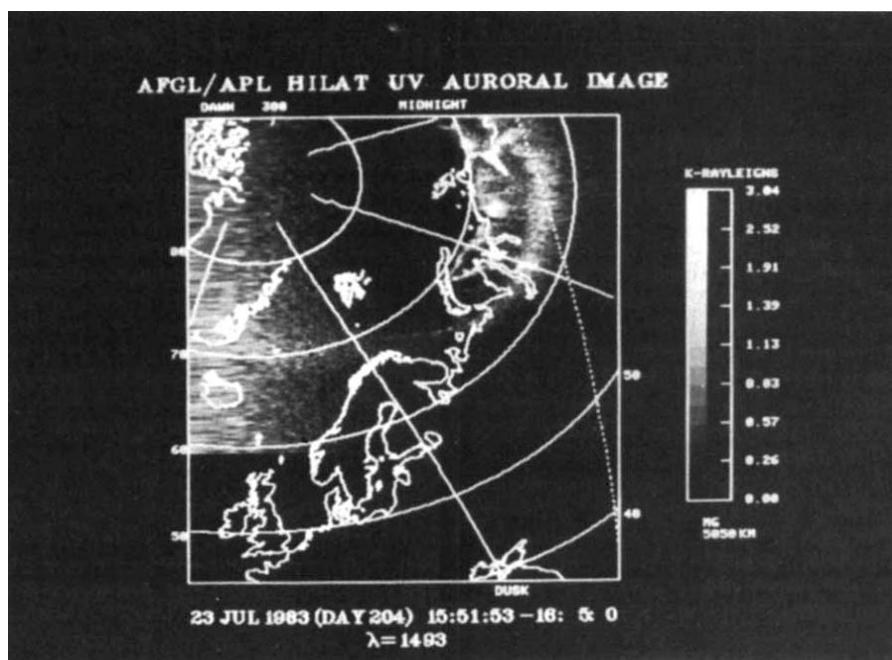
The aurora cannot be seen in daylight because the brightness of scattered light (the blueness of the sky) overwhelms the much fainter light from the aurora. At ultraviolet wavelengths (below 200 nm), however, the background of scattered light is reduced both because the incoming solar radiation is weaker and because the radiation is totally absorbed by the atmosphere before penetrating it or being reflected from it.

Molecules excited by the impact of auroral electrons emit light at ultraviolet wavelengths, notably N_2 in molecular bands between 132.5 and 150 nm, and atomic oxygen with transitions at 130.4 and 135.6 nm. These emissions cannot be seen from the ground because they too are absorbed by the atmosphere. They can, however, be seen from above by instruments carried on satellites because they occur at such a height that the light can escape upwards without much attenuation.

The first auroral images in ultraviolet light, obtained in full sunlight by the American polar-orbiting spacecraft Hilat, have just been published (Meng & Huffman *Geophys. Res. Lett.* 4, 135; 1984). As the instrument looks down from a height of 820 km it scans a strip 5,000 km long stretching from horizon to horizon across the satellite track. The forward movement of the satellite builds up a picture from a series of parallel lines just as a television tube does. The intensity in a band only 3 nm wide is measured by a spectrometer which covers the range of wavelengths from 110 to 190 nm. This narrow bandwidth improves the signal-to-background ratio when the spectrometer is set on the most prominent features in the

auroral spectrum. The proof of the technique is in the picture below which shows a typical auroral structure, familiar from the nightside images.

Now that the restrictions on auroral images imposed by sunlight have been removed, the exciting possibility emerges of obtaining pictures of the aurora simultaneously at both ends of a geomagnetic field line. This would require recording by spectrometers on at least two satellites. A survey of the extent to which the aurora is conjugate would provide a great deal of information about the processes responsible. □



Hilat auroral imagery over sunlit summer polar region. The nightside, under full sunlight in summer, of the auroral oval is over the arctic coast of the Soviet Union. The day-night terminator on the Earth's surface is indicated by the dotted line. The picture is in geomagnetic coordinate. The land mass above 80° geographic latitude is deleted in the picture.

Plant science

Molecular studies forge ahead

from John Ingle

EIGHT years and four meetings have passed since the first NATO/FEBS Advanced Study Institute provided a focus for the then fledgling science of plant molecular biology. The progress can honestly be described as dramatic. Research has progressed from the initial problems of DNA isolation and cloning, through nucleotide sequence analysis and vector construction to the use of site-directed mutagenesis for detailed study of regulatory and structural gene sequences. Physiological problems are now routinely addressed via the modulation of gene expression at both RNA and protein levels, and the transfer and efficient expression of tissue-regulated genes is an established fact. The impact and potential of molecular manipulations on the improvement of plants has attracted commercial interest in agricultural opportunities, a point amply illustrated by the strength and vigour of biotechnology com-

pany participation at a recent meeting*.

The initial focus of plant molecular biology on the chloroplast has largely continued, producing a detailed analytical description of its genome. Functional characterization of the genome has been less successful, depending largely on the use of heterologous *in vitro* systems, such as *Escherichia coli*. The development of an homologous transcription system and characterization of the RNA polymerase activities responsible for expression of chloroplast genes, together with the identification of chloroplast promoter elements, represents a major advance (W. Gruissem, University of California, Berkeley), but a chloroplast transformation system is still urgently needed.

For nuclear genes a welcome main trend

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*'Molecular form and function of the plant genome', NATO Advanced Studies Institute FEBS Advanced Course, 4-14 July, Renesse, The Netherlands.

is the increase in diversity of genes being studied and the improved detail of expression studies. The small subunit of ribulose biphosphate carboxylase still dominates the field, offering an excellent experimental system for the study of such crucial problems as transport of proteins across membranes, regulation in response to light and expression of individual genes within a gene family. The complexity of gene expression, particularly at the post-transcriptional level, was indicated from a detailed study on the sulphur-rich albumin in pea seeds (T.J.V. Higgins, CSIRO, Canberra). In plants grown in sulphur-deficient conditions, accumulation of albumin is severely reduced and there is a low level of messenger RNA. This is due to destabilization of transcripts, which are synthesized at approximately the same rate as in control plants.

The complexity of the mitochondrial genome far exceeds that of the chloroplast, both in genetic and structural terms (D. Lonsdale, Plant Breeding Institute, Cambridge). The genome consists principally of circular DNA, and a master chromosome of 570 kilobase pairs (kbp) has been described in maize. The presence of several different repeated sequences within this circular molecule can result, however, in dramatic genome rearrangements through recombination, producing a variety of circular mitochondrial chromosomes. Mitochondria also possess small plasmids or episomes, which may be circular or linear. In the case of the linear episomes in the S male sterile cytoplasm, the terminal inverted repeats of the episomes share sequences with the circular chromosome such that recombination results in the linearization and fragmentation of the circular genome. The S-type mitochondrial genome therefore undergoes a dynamic flux between circular and linear chromosomal forms, and it is tempting to give these DNA rearrangements functional significance, such as control of the male sterile phenotype.

Functional modification of the genome appears to be necessary for differentiation of nitrogen-fixing heterocysts in *Anabaena* (R. Haselkorn, University of Chicago). In vegetative non-fixing cells the *nif K* and *nif D* genes are separated by 10 kbp. This DNA is excised during the early stages of heterocyst differentiation, thereby bringing the genes into close proximity, and remains in the cell as a 10 kbp circle. These examples of mitochondrial DNA and *nif* DNA rearrangements, together with the genomic splicing mechanisms associated with immunoglobulin expression in animals, suggest that genome rearrangement may be a general feature of differentiation and development.

Commercial interest in improving plant varieties by genetic manipulation was well illustrated by excellent contributions on herbicide resistance. The 32,000-molecular weight ubiquinone-binding protein of photosystem II in the chloroplast was

probably the most discussed protein of the meeting. A single amino acid change confers resistance to triazine herbicides, but the lack of a chloroplast transformation system has so far prevented its exploitation, except by cell fusion technology. The biochemical mechanisms involved in resistance to other herbicides, such as glutathione-S-transferase for chloroacetamides or triazines, 3-phosphoshikimate 1-carboxyvinyltransferase for glyphosate or acetolactate synthase for sulphonylureas, have been elucidated (R. Fraley, Monsanto, St Louis; L. Comai, Calgene, Davis; and S.C. Falco, Du Pont, Wilmington, respectively), and genes conferring resistance have been isolated and characterized from plants, bacteria and yeast. Some of these genes have been transferred to plants, but so far no resistant plants have been reported.

The successful transfer of the phaseolin gene from *Phaseolus* to sunflower has been

followed up by transfer into tobacco. Regenerated tobacco plants have given the first clear indication of developmental regulation of a transferred gene since phaseolin, a seed storage protein, was produced in tobacco seeds but not in leaves, and the single copy of the gene appeared to be expressed to a similar level to that in *Phaseolus* seed (T.C. Hall, Agrigenetics, Madison). These results, together with a much improved 'leaf disk dip' for transformation of plant tissue (R. Fraley, Monsanto, St Louis) and the success of monocotyledon transformation by *Agrobacterium tumefaciens* (P. Hooykaas, University of Leiden), fully support the practical potential that has been envisaged for foreign gene expression in agricultural plants. □

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Growth factors

The epidermal growth factor receptor gene and its product

from Tony Hunter

THE establishment of a direct mechanistic link between growth control and tumorigenesis has been a long-standing pipe-dream, but, within the last year, two such connections have been made. In both cases the connection is between the product of a known viral oncogene and a known cellular growth factor or its receptor. Late last year, the product of the *v-sis* gene was found to be closely related to platelet-derived growth factor (PDGF); and more recently, a remarkable similarity has been demonstrated between the product of the *v-erb-B* oncogene of avian erythroblastosis virus (AEV) and the receptor for epidermal growth factor (EGF)¹. This homology and some partial protein sequence data for the EGF receptor have now enabled three groups to isolate cDNA clones for the EGF receptor mRNA and thus extend the characterization of the EGF receptor²⁻⁴.

All three groups used as their primary source of EGF receptor mRNA the A431 human carcinoma cell line, which expresses 10–50 times more EGF receptors on its surface than most other cell lines do. The cloning of the EGF receptor mRNA has now helped to elucidate the normal structure and function of the receptor, its relationship to the *v-erb-B* oncogene product and several abnormalities associated with its expression in A431 cells.

EGF receptor protein structure

The EGF receptor was already known to be a 170,000–180,000 molecular weight (170–180K) glycoprotein with an intrinsic tyrosine-specific protein kinase which is

stimulated upon binding EGF. From the primary amino acid sequence predicted from the nucleotide sequences of cDNA clones covering the entire coding region, the following picture of the EGF receptor now emerges (ref. 2 and see the figure). A signal peptide of 24 amino acids is cleaved from a precursor to yield a mature protein of 1,186 amino acids. The receptor can be divided figuratively into two parts: an NH₂-terminal region of 621 amino acids and a COOH-terminal domain of 542 amino acids, separated by a run of 26 preponderantly hydrophobic amino acids, which has been proposed to form a membrane-spanning domain^{2,3}. The putative transmembrane domain is bounded on the cytoplasmic side by an extremely basic (9/15 residues) sequence, a feature which is common to many membrane-spanning segments.

From its sequence one would deduce that the N-terminal region is the external EGF-binding domain; it contains 51 cysteine residues, many of which must be involved in disulphide bonds, and 12 potential glycosylation sites, a number which fits well with the experimentally determined number of oligosaccharide chains⁵. The cysteines are clustered in two regions, each of about 170 amino acids (positions 132–313 and 446–612), which show similarities in the spacing of individual cysteines². These might form a two-lobed structure with an EGF-binding cleft. This 'duplicated' structure could be involved in ligand-mediated receptor oligomerization.

The C-terminal region has strong sequence homology with the predicted

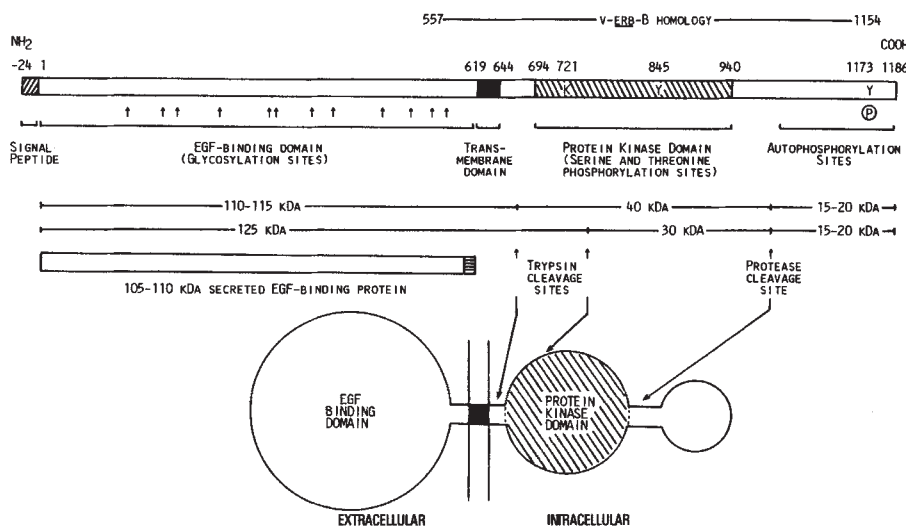
v-erb-B gene product over a stretch of 250 amino acids starting at residue 694, about 50 amino acids from the proposed transmembrane domain. This region also shows homology to the catalytic domains of other protein kinases including the members of the *src* gene family of tyrosine-phosphorylating protein kinases. Thus it seems likely that this region comprises a cytoplasmic domain responsible for the tyrosine protein kinase activity of the receptor. The final 240 amino acids at the C-terminus of the EGF receptor show no homology with known proteins.

Gene structure and expression

Based on the sizes of the restriction enzyme fragments in placental DNA homologous with the various cDNA clones it can be deduced that the human EGF receptor gene is a large gene, greater than 50 kilobases (kb) in size². All three groups find that the receptor gene is amplified in A431 cells, from 3- to 100-fold depending on the clone of cells analysed^{2,4,6}. The amplification could in principle be responsible for the high level of EGF receptors found on these cells. Indeed, there does appear to be some correlation between the degree of amplification and the expression of EGF receptor both at the mRNA level and at the protein level^{3,4}. The amplification may be associated with a translocation involving chromosome 7, to which the EGF receptor gene has been mapped^{7,8}, although the amplification of the gene is greater than that expected from the total number of copies of both normal and translocated chromosome 7 found in A431 cells⁹. The amplification does not seem to be a direct consequence of the translocation event since cells lacking the translocated chromosome have an amplified EGF receptor gene⁴. The structure of the amplified genes is similar to that of the normal gene, although there are minor differences^{2,3}.

The EGF receptor gene is expressed in the form of at least two mRNAs, with sizes of about 10 and 6 kb (refs 2-4). The exact relationship between these mRNAs is not yet known. The 6 kb RNA predominates but the ratio of the two RNAs differs between cell types. The structures of the overlapping cDNA clones² prove that the 6 kb RNA can encode the EGF receptor, but whether this is the case for the 10 kb RNA is uncertain. The two RNAs could well have similar 5' ends and differ through virtue of alternate splicing pathways or poly(A) addition sites such that the 10 kb RNA carries a longer 3'-untranslated region.

In A431 cells a third EGF receptor-related mRNA is detected with a size of about 3 kb. The origin of this RNA is unclear but it is more common than the 6 kb RNA and might therefore arise from the amplified copies of the receptor gene, possibly as the result of an abnormal splicing event or some minor change in gene structure. This small RNA is all the more intriguing because its sequence



Relationship between the *v-erbB* gene (top), EGF receptor gene (middle) and EGF receptor protein (bottom), shown in linear form and as it might be organized in spanning the membrane of a cell.

corresponds to just the N-terminal half of the EGF receptor, including most of the EGF-binding region and a tail of 18 amino acids from an unknown source. Because the predicted protein lacks the transmembrane domain, it could well be secreted; and in fact, A431 cells have been observed to secrete just such an EGF-binding activity in the form of a 105-110K glycoprotein^{5,10}. So far the 3 kb RNA and secreted protein have been detected only in A431 cells, and it will be interesting to see whether they are made by any normal cell type.

Does the more extensive knowledge of the EGF receptor gene allow us to decide whether it is the *c-erb-B* gene as has been proposed? When amino acids 557-1,154 of the EGF receptor are compared with the *v-erb-B* gene product an overall homology of about 85 per cent is found, which rises to a striking 97 per cent homology in the protein kinase domain. The main difficulty in deciding on this basis whether the two genes are identical is that one is comparing the human EGF receptor with a chicken-derived oncogene potentially carrying point mutations. Human sequences homologous to the *v-erb-B* gene have been cloned¹¹. They presumably arose from the *c-erb-B* gene, but no nucleotide sequence data are available to show whether it is a genuine identity rather than a close relationship. Chromosome-mapping results are consistent with the human *c-erb-B*-related sequence (chromosome 7 between 7p13 and 7q22)¹² and the human EGF receptor gene (chromosome 7 between 7p22 and 7qter)^{7,8} being one and the same. On balance, therefore, it seems likely that the *c-erb-B* gene is the EGF receptor gene, but it is still formally possible that there are two related genes on chromosome 7 with homology in the protein kinase domain.

EGF receptor protein kinase

What is the evidence that the protein kinase activity of the receptor is located where the primary sequence predicts? There are several reports indicating that a fragment

of 15-20K can be clipped proteolytically from the cytoplasmic domain of the EGF receptor leaving a 150K fragment which retains EGF-stimulated protein kinase activity. In the light of the sequence data, the 15-20 K fragment probably corresponds to the C-terminal region, which may be connected to the protein kinase domain by an exposed linker accessible to proteases. More extensive tryptic proteolysis of the purified receptor either in solution or bound to antibody gives rise to 30-40K and 110-125K fragments^{5,13,14}, presumably arising from the 150K C-terminally truncated receptor. The 110-125K fragment contains the EGF-binding site and all of the glycosyl side chains¹³. A report in this issue of *Nature* by Basu *et al.*¹⁴ shows that the 40K fragment generated by trypsin treatment of receptor in solution retains about 30 per cent of the tyrosine protein kinase activity of the intact receptor. By contrast, the 30K fragment, which is the main species liberated by trypsin from EGF receptor bound to antibody, seems to lack tyrosine kinase activity¹³. The existence of alternate protease cleavage patterns for receptor in solution and bound to antibody might explain this difference.

The 40K fragment can both 'autophosphorylate' and phosphorylate exogenous substrates, but this activity is no longer regulated by EGF, nor does the fragment bind EGF¹⁴. The 40K fragment can be modified by the ATP analogue, 5'-*p*-fluorosulphonylbenzoyl adenosine (FSBA), concomitant with the loss of protein kinase activity, as had previously been reported for the holoreceptor. In the case of the archetypal tyrosine protein kinase, pp60^{v-src}, FSBA inactivation is due to covalent modification of lysine 295 (ref. 15). This lysine is 16 residues downstream of the Gly-X-Gly-X-X-Gly sequence believed to be involved in nucleotide binding. Lysine 295 of pp60^{v-src} is homologous to the ATP-binding lysine 71 in the cyclic AMP-dependent protein kinase and would correspond to lysine 721

in the EGF receptor or lysine 165 in the *v-erb-B* protein, both of which are 21 residues downstream of a Gly-X-Gly-X-X-Gly sequence. The likely location of the ATP-binding site, together with the results of Basu *et al.*, provide strong experimental support for the predicted location of the protein kinase domain within the EGF receptor. It is interesting to note that two of the retroviral tyrosine protein kinases can be cleaved by proteases to generate 30–40K fragments which retain tyrosine protein kinase activity^{16,17}. Perhaps all tyrosine protein kinases which are members of the *src* family will prove to have such a protease-resistant catalytic domain.

Receptor phosphorylation

The sites of phosphorylation in the EGF receptor are of obvious interest because of intimations that its protein kinase activity may be regulated by phosphorylation. Based on sequence studies of phosphopeptides, Downward *et al.*¹⁸ report in this issue of *Nature* that at least three out of four major 'autophosphorylation' sites are in the C-terminal 15–20K tail, suggesting an attractive model in which the C-terminal domain, by virtue of a flexible connection to the rest of the molecule, might be able to fold over and interact with the catalytic site.

Tyrosine 1,173, one of the autophosphorylation sites, corresponds to the only tyrosine detectably phosphorylated in intact cells in response to EGF^{18,19}. Occupation of this site correlates with increased phosphotransferase activity towards exogenous substrates, perhaps because it results in the displacement of the C-terminal domain from the active site, thus allowing access to exogenous substrates. Two other autophosphorylation sites have been mapped close to the C-terminus (residues 1,068 and 1,148), but since these are only weakly phosphorylated in the cell, their significance is uncertain.

The C-terminal region of the EGF receptor, containing tyrosine 1,173, is missing from the *v-erb-B* genes of both strains of AEV. It is not clear whether the loss of this potentially regulatory region is a *sine qua non* for the oncogenic activation of the *c-erb-B* gene or whether it is simply a consequence of the recombination mechanism by which this gene was coopted. Examination of the structure of *c-erb-B* genes which have been activated by viral long-terminal-repeat promoter insertion²⁰ may well provide the answer to this question.

The retroviral tyrosine protein kinases all have a site of tyrosine phosphorylation in the catalytic domain, which can be autophosphorylated *in vitro*. In pp60^{v-src} it is tyrosine 416, which aligns with an homologous tyrosine that is phosphorylated in the other retroviral tyrosine kinases. Tyrosine 416 of pp60^{v-src} is homologous to tyrosine 845 within the protein kinase domain of the EGF receptor, yet apparently tyrosine 845 is not phosphorylated to a measurable extent in intact cells.

The effect of this tyrosine phosphorylation on the activity of the retroviral protein kinases is variable, being minimal in the case of pp60^{v-src} (ref.21), but of greater consequence for P140^{src-fps} (ref.22). Regardless of the functional significance of this phosphorylation it is somewhat surprising that autophosphorylation of tyrosine 845 in the EGF receptor is not observed.

In addition to tyrosine 1,173, the EGF receptor also has several sites of threonine and serine phosphorylation which are localized predominantly in the 30–40K proteolytic fragments and thus lie in the protein kinase domain¹³. The provenance of the protein kinases responsible for these phosphorylations is uncertain, nor is it known whether these modifications modulate receptor function. The EGF receptor can be phosphorylated *in vitro* by the cyclic AMP-dependent protein kinase^{23,24}, but this does not appear to alter its tyrosine protein kinase activity and there is no evidence that it is physiologically significant.

Two recent reports have indicated that the EGF receptor is also a substrate for the Ca²⁺/phospholipid-dependent diacylglycerol-activated serine/threonine-specific protein kinase, C-kinase^{25,26}. C-kinase appears to be the major receptor for the phorbol ester tumour promoters, such as 12-*O*-tetradecanoylphorbol-13 acetate (TPA), being activated upon binding phorbol esters. TPA enhances serine and threonine phosphorylation of EGF receptor in A431 cells, and at least one new site of threonine phosphorylation is evident^{25,26}. This site is also phosphorylated when purified EGF receptor is incubated with purified C-kinase²⁵. C-kinase-mediated phosphorylation of the EGF receptor in A431 cells leads to a diminished ability of EGF to stimulate its tyrosine protein kinase activity^{25,27}. It is therefore intriguing that under some circumstances the same site is phosphorylated following EGF treatment²⁶. Since EGF is known to increase the turnover of phosphatidylinositol, leading to formation of diacylglycerol, this effect of EGF may be mediated indirectly through an activation of C-kinase resulting from increased diacylglycerol production. Possibly the normal role of this phosphorylation is to act as a feedback mechanism to attenuate the response of the EGF receptor. Increased phosphatidylinositol turnover is a rather general effect of mitogen treatment, so it will be interesting to find out whether similar mechanisms exist for other growth factor receptors.

In fibroblasts TPA treatment leads to decreased EGF binding due to changes in either binding affinity or receptor number, while in A431 cells a class of very high-affinity receptors is lost. This effect, together with that on the tyrosine protein kinase activity of the receptor, indicates that phosphorylation of the cytoplasmic

domain of the receptor by C-kinase can modulate communication between the extracellular EGF-binding domain and the protein kinase domain. From our own data, reported in this issue of *Nature*²⁸, the major site of threonine phosphorylation for C-kinase in the A431 cell EGF receptor is threonine 654, which lies in the basic region nine residues from the cytoplasmic end of the proposed transmembrane domain. Thus this threonine is situated between the extracellular domain and the protein kinase domain in an ideal position to affect interaction of the two domains.

An important issue raised by our new knowledge of the structure of the EGF receptor is how EGF binding transmits signals to the cytoplasm. It is not clear whether a protein chain which only traverses the plasma membrane once could transmit an allosteric signal. Potentially, oligomerization of the receptor in the membrane could be required, although one has to explain how EGF stimulates the phosphotransferase activity of the EGF receptor in solution. Another consideration is that the *v-erb-B* gene product, which lacks the majority of the extracellular domain, does not display overt tyrosine protein kinase activity. This implies that simple removal of the regulatory domain does not lead to constitutive activation of the catalytic domain. A comparison of the structures of the other receptors with inducible tyrosine protein kinase activities, such as the PDGF and insulin receptors, may give us a clue to the mechanism of signal transduction. The cloning and sequencing of these receptors is eagerly awaited. □

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Malignant cell lines — why use APUDoma?

SIR — Several investigators have referred to our cell lines, COLO 320 and 321 as APUDomas (meaning “containing amine precursor uptake and decarboxylase activities”). These cell lines were derived from a carcinoma of the colon and are interesting because of their replication of *c-myc* oncogene¹. Our description² of the cell lines included a list of cell products. The detection of several peptide hormones was subsequently interpreted by others as evidence that the cell line should be labelled “APUDoma”.

Many diverse malignancies have been “frozen” in a stage of differentiation which results in activation of the genes for one or multiple peptide hormones³. There are probably malignant forms of every single kind of cell that could be labelled “APUDoma”.

It seems reasonable that cell line designations should not be altered but that unique aspects of the cells should be noted if relevant to the work being described.

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Mechanism for heritability of intelligence

SIR — I wish to suggest a partial answer to the important question of what mechanism in the central nervous system may account for the heritability of intelligence¹.

Although we are far from having a generally agreed definition of intelligence, let alone an understanding of the mechanism responsible, I believe that even now we can specify one necessary neurophysiological aspect of the variation in degree of intelligence and show that it has an appreciable degree of genetic determination. Whatever intelligence is, it must require communication, by nerve impulses, among different regions of the brain. These impulses are transmitted along nerve fibres and across synapses with the participation of many different proteins (including enzymes) and other molecules, such as neurotransmitters (which are synthesized by specific enzymes). These ‘transmission proteins’, like all proteins, are composed of polypeptide chains which are direct gene products.

There is great genetic diversity among humans (within races); half or more of structural gene loci are polymorphic, that is, they have two or more common alleles (genes) at that locus². We consequently expect appreciable genetic variability

among individuals for the genes specifying these ‘transmission proteins’. In addition to this direct genetic variation in the structure, and hence the activity of these proteins, there is a second kind of genetically specified variation: differences, among normal individuals, in the amount (concentration) of each specific protein. In particular, whenever enzyme concentrations, for example, dopamine- β -hydroxylase and catechol-*O*-methyltransferase (which, respectively, synthesize and metabolize the neurotransmitter noradrenaline) have been studied genetically, they have been found to be under appreciable genetic control³.

This genetic variability, both in structure and amount of transmission proteins is, of course, heritable from parent to offspring. This variability will affect nerve impulse transmission in the brain to some degree and in a variety of ways, such as in speed and accuracy. It therefore sets limits on information processing rates. Data from mice support this interpretation, directly showing heritability of nerve conduction velocity^{4,5} and synaptic transmission time⁶. This genetic control of information processing is, I submit, a sufficient basis for asserting that there is significant genetic determination of variation in human intelligence.

I do not suggest of course, that such a mechanism would operate in isolation. I believe that in all societies, environment (in the broadest sense, beginning prenatally) also has very important effects (positive and negative) on human intelligence.

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Atmospheric humidity in the nuclear winter

SIR — Recent calculations^{1,2} and discussion of the possibility of a ‘nuclear winter’ consequent to widespread nuclear war may have ignored an important effect of atmospheric humidity. The nuclear winter is calculated² to be severe for wars in the late spring or summer. In those seasons, at most assumed targets the dew point is quite high, usually no less than 55°F (13°C). Soot from urban or forest fires would be produced intimately mixed in this humid air.

In order to reach the ‘nuclear winter’ state this air is chilled. That portion of it which rises to the upper troposphere or higher reaches ambient temperatures when its rise stabilizes, in an hour or less; only on a much longer time scale does solar heating

rewarm it. The remaining sooty air is below the warming influence of sunlight, and it cools to the low surface air temperatures calculated for the nuclear winter. In each case the air is cooled to a temperature at which the saturation water vapour pressure is far below the initial vapour content. The resulting condensate efficiently scavenges the soot aerosol, as in the precipitation cleaning of urban smog and the black rain observed after firestorms; precipitation removes both water and soot from the atmosphere.

Scavenging is unavoidable: in nuclear winter calculations the air begins warm and humid and ends cool and dry, a process which must efficiently purge it of its water vapour and aerosols. This effect has been ignored in the published calculations^{1,2} because they begin by assuming a distribution of soot in the troposphere, and do not calculate condensation and scavenging. A quantitative understanding will require detailed calculation of rising sooty plumes and of condensation and scavenging both there and in the cooling troposphere.

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DNA can be a selfish parasite

SIR — Evans, recently pointed out a ‘fatal flaw’ in all evolutionary discussions of selfish or parasitic DNA. The problem, as outlined by Evans, is that any element which imposes any cost on its host will be eliminated by natural selection; this selection pressure can be counteracted only by a mechanism for horizontal transfer (presumably virus-like infection of cells) or by a high rate of *de novo* generation of new elements. The latter condition is highly unlikely and the former would imply that parasitic DNA is merely a new label applicable only to conventional viruses. I cannot agree with these conclusions.

I have already explored² in some detail the questions raised by Evans and the main conclusion is that parasitic DNAs can potentially spread very rapidly in natural populations despite the fact that they may also have significant negative effects on host fitness. A mechanism of horizontal spread is not necessary provided there is biparental inheritance of host genomes. This criterion is met in all sexually-reproducing, out-breeding higher eukaryotes. Parasitic elements capable of duplicate transposition are able to ‘colonize’ new chromosomes at zygote formation. Thus, the elements can spread to new genomes by a means other than horizontal transfer. The general prediction of this model is that we expect a higher level of such parasites within the genomes of out-breeding higher

eukaryotes than within the genomes of asexually reproducing prokaryotes. The experimental evidence is consistent with this prediction.

Other theoretical studies³⁻⁵ have incorporated negative selection into models of selfish DNA and, recently, such a model⁶ was applied explicitly to the case of transposable P elements in *Drosophila*. P elements have a demonstrable negative effect on host fitness, yet they have spread rapidly within natural populations⁷.

In conclusion, I believe that the remarks of Evans¹ are very pertinent to the question of parasitic DNA in some genomes but the situation is less restrictive than he describes for the case of the higher eukaryotes. I would say that parasitic DNA is not only possible but highly probable in these latter organisms. The evolutionary pressures on this DNA would indeed be of the type outlined by Doolittle *et al.*⁸.

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Primroses and self-fertilization

SIR — Bodmer's recent letter¹ concerning homostyle primroses raises some scientific issues on which we would like to comment. First, he suggests that it is important to explain why the self-fertile homostyle variant is confined to a few English populations; his model of a low rate of self-fertilization and a small viability disadvantage to homostyles^{1,2} is intended to provide such an explanation in view of the absence of evidence for the large viability disadvantage of homozygous homostyles postulated by Crosby³. There is, however, no direct evidence that the primrose populations containing homostyles are tending towards a stable polymorphic equilibrium, as Crosby and Bodmer assume. As was previously pointed out⁴, the polymorphism for homostyles in *Primula vulgaris* is a highly exceptional situation; most species of *Primula* are, of course, distylous and self-incompatible, but there are many in which homostyly has become fixed^{5,6}. The same is true of other groups of distylous plants^{7,8}. It is thus entirely possible that *P. vulgaris* is merely in a state of transient polymorphism, and that the homostyle variant is on its way to fixation, at least locally⁴. The model of a conflict between the advantage of fertility assurance of a self-fertile form and the disadvantage of inbreeding depression to

its progeny can explain why homostyles become established only in some species or in some geographical areas of a species^{4,9}; the biogeographic data on the distribution of homostyly are consistent with this model^{4,6,8}.

Second, Bodmer suggests that the selfing rate of homostyles may vary in time and space, so that our estimate⁹ of a value of 0.92 for a single population in 1982 is not necessarily representative. We certainly agree with this, but would like to point out that there are two other independent estimates of the selfing rate for natural populations which give values of 0.93 (refs 10, 11); within the limits of sampling error, these estimates agree. Richards¹² has made clear the difficulties in interpreting Bodmer's low selfing rate estimate^{2,13} for garden material, and in extrapolating this to natural populations. (As noted by Crosby¹⁰, this estimate excluded data from a year (1955) in which the selfing rate was apparently unity). The weight of the empirical evidence is, we feel, in favour of a high selfing rate for homostyle primroses in nature.

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Herpes simplex transforming fragments

SIR — In the past twelve months, the subject of viral genes involved in immortalization and tumorigenic transformation has been reviewed at least three times in the News and Views section of *Nature*. Every time when reference was made to DNA tumour viruses, it was stated that genetically distinct functions for immortalization and malignant transformation have been demonstrated for two DNA viruses, polyoma virus and adenovirus. Mention was never made of the separable immortalizing and tumorigenic functions that have been identified in the genome of the human DNA virus herpes simplex type 2 (HSV-2).

The first report of escape from senescence and neoplastic transformation of early passage, nonestablished Syrian hamster embryo cells by a defined subgenomic fragment of HSV-2 DNA appeared in the spring of 1980¹. Last autumn evidence was published² for the existence of two separate genetic functions within this defined fragment. One

subfragment derived from the 64% left-hand end was shown to cause immortalization, whereas the other subfragment from the 36% right-hand end was demonstrated to cooperate with the left-hand subfragment in mediating tumorigenic transformation. What makes the HSV-2 data especially interesting is that the immortalizing DNA segment contains a region that exhibits homology to an interspersed middle-repetitive DNA sequence in mammalian cells^{2,3}. I should like to bring these reports to the attention of *Nature* and its readers.

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Chinese sources no guide to Geminga

SIR — Pannekoek, in his study of Ptolemy¹, remarks "In studying the history of astronomy we are not learning something about the stars, but something about the astronomers". This statement is true for the Chinese records as well. The Chinese records are notorious for their inaccuracy and unreliability², and modern astronomers have been too keen to identify new sources with ancient recorded phenomena. Kukarkin *et al.*³ list a good many identifications of ancient Chinese "nova" recordings with modern counterparts, and note that the vast majority of these is unfounded, and many (notably radiosources) are demonstrably wrong.

The reality of a phenomenon chronicled by the Chinese often has to be doubted², and the position is always uncertain (a famous example is the wrong position for the Crab nebula⁴). Rather than contribute to the spread of spurious identifications in the literature — once in existence, they die hard — Editors of scientific journals⁵ would do well to show more restraint in publicizing such identifications. Bignami *et al.*⁶ are justified in mentioning the Chinese record of AD 437; but its identification with Geminga can only be accepted if in addition to the positional coincidence (of necessity inaccurate) a much more precise temporal coincidence is established.

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Cellular automata as models of complexity

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Natural systems from snowflakes to mollusc shells show a great diversity of complex patterns. The origins of such complexity can be investigated through mathematical models termed 'cellular automata'. Cellular automata consist of many identical components, each simple, but together capable of complex behaviour. They are analysed both as discrete dynamical systems, and as information-processing systems. Here some of their universal features are discussed, and some general principles are suggested.

It is common in nature to find systems whose overall behaviour is extremely complex, yet whose fundamental component parts are each very simple. The complexity is generated by the cooperative effect of many simple identical components. Much has been discovered about the nature of the components in physical and biological systems; little is known about the mechanisms by which these components act together to give the overall complexity observed. What is now needed is a general mathematical theory to describe the nature and generation of complexity.

Cellular automata are examples of mathematical systems constructed from many identical components, each simple, but together capable of complex behaviour. From their analysis, one may, on the one hand, develop specific models for particular systems, and, on the other hand, hope to abstract general principles applicable to a wide variety of complex systems. Some recent results on cellular automata will now be outlined; more extensive accounts and references may be found in refs 1-4.

Cellular automata

A one-dimensional cellular automaton consists of a line of sites, with each site carrying a value 0 or 1 (or in general 0, ..., $k-1$). The value a_i of the site at each position i is updated in discrete time steps according to an identical deterministic rule depending on a neighbourhood of sites around it:

$$a_i^{(t+1)} = \phi[a_{i-r}^{(t)}, a_{i-r+1}^{(t)}, \dots, a_{i+r}^{(t)}] \quad (1)$$

Even with $k=2$ and $r=1$ or 2, the overall behaviour of cellular automata constructed in this simple way can be extremely complex.

Consider first the patterns generated by cellular automata evolving from simple 'seeds' consisting of a few non-zero sites. Some local rules ϕ give rise to simple behaviour; others produce complicated patterns. An extensive empirical study suggests that the patterns take on four qualitative forms, illustrated in Fig. 1:

- (1) disappears with time;
- (2) evolves to a fixed finite size;
- (3) grows indefinitely at a fixed speed;
- (4) grows and contracts irregularly.

Patterns of type 3 are often found to be self-similar or scale invariant. Parts of such patterns, when magnified, are indistinguishable from the whole. The patterns are characterized by a fractal dimension⁵; the value $\log_2 3 \approx 1.59$ is the most common. Many of the self-similar patterns seen in natural systems may, in fact, be generated by cellular automaton evolution.

Figure 3 shows the evolution of cellular automata from initial states where each site is assigned each of its k possible values with an independent equal probability. Self-organization is seen: ordered structure is generated from these disordered initial states, and in some cases considerable complexity is evident.

Different initial states with a particular cellular automaton rule yield patterns that differ in detail, but are similar in form and statistical properties. Different cellular automaton rules yield very different patterns. An empirical study, nevertheless, suggests that four qualitative classes may be identified, yielding four characteristic limiting forms:

- (1) spatially homogeneous state;
- (2) sequence of simple stable or periodic structures;
- (3) chaotic aperiodic behaviour;
- (4) complicated localized structures, some propagating.

All cellular automata within each class, regardless of the details of their construction and evolution rules, exhibit qualitatively similar behaviour. Such universality should make general results on these classes applicable to a wide variety of systems modelled by cellular automata.

Applications

Current mathematical models of natural systems are usually based on differential equations which describe the smooth variation of one parameter as a function of a few others. Cellular automata provide alternative and in some respects complemen-

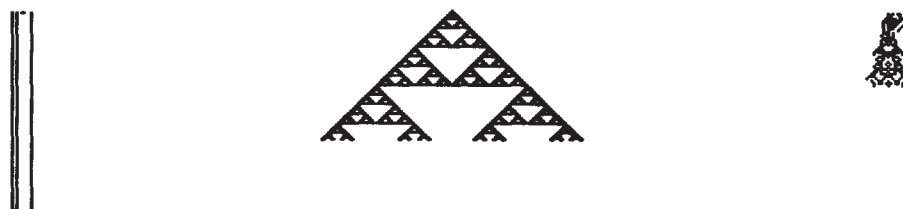


Fig. 1 Classes of patterns generated by the evolution of cellular automata from simple 'seeds'. Successive rows correspond to successive time steps in the cellular automaton evolution. Each site is updated at each time step according to equation (1) by cellular automaton rules that depend on the values of a neighbourhood of sites at the previous time step. Sites with values 0 and 1 are represented by white and black squares, respectively. Despite the simplicity of their construction, patterns of some complexity are seen to be generated. The rules shown exemplify the four classes of behaviour found. (The first three are $k=2$, $r=1$ rules with rule numbers¹ 128, 4 and 126, respectively; the fourth is a $k=2$, $r=2$ rule with totalistic code² 52.) In the third case, a self similar pattern is formed.

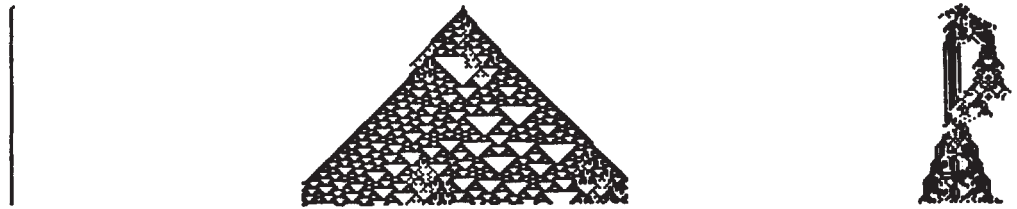


Fig. 2 Evolution of small initial perturbations in cellular automata, as shown by the difference (modulo two) between patterns generated from two disordered initial states differing in the value of a single site. The examples shown illustrate the four classes of behaviour found. Information on changes in the initial state almost always propagates only a finite distance in the first two classes, but may propagate an arbitrary distance in the third and fourth classes.

tary models, describing the discrete evolution of many (identical) components. Models based on cellular automata are typically most appropriate in highly nonlinear regimes of physical systems, and in chemical and biological systems where discrete thresholds occur. Cellular automata are particularly suitable as models when growth inhibition effects are important.

As one example, cellular automata provide global models for the growth of dendritic crystals (such as snowflakes)⁶. Starting from a simple seed, sites with values representing the solid phase are aggregated according to a two-dimensional rule that accounts for the inhibition of growth near newly-aggregated sites, resulting in a fractal pattern of growth. Nonlinear chemical reaction-diffusion systems give another example^{7,8}: a simple cellular automaton rule with growth inhibition captures the essential features of the usual partial differential equations, and reproduces the spatial patterns seen. Turbulent fluids may also potentially be modelled as cellular automata with local interactions between discrete vortices on lattice sites.

If probabilistic noise is added to the time evolution rule (1), then cellular automata may be identified as generalized Ising models^{9,10}. Phase transitions may occur if ϕ retains some deterministic components, or in more than one dimension.

Cellular automata may serve as suitable models for a wide variety of biological systems. In particular, they may suggest mechanisms for biological pattern formation. For example, the patterns of pigmentation found on many mollusc shells bear a striking resemblance to patterns generated by class 2 and 3 cellular automata (see refs 11, 12), and cellular automaton models for the growth of some pigmentation patterns have been constructed¹³.

Mathematical approaches

Rather than describing specific applications of cellular automata, this article concentrates on general mathematical features of their behaviour. Two complementary approaches provide characterizations of the four classes of behaviour seen in Fig. 3.

In the first approach², cellular automata are viewed as discrete dynamical systems (see ref. 14), or discrete idealizations of partial differential equations. The set of possible (infinite) configurations of a cellular automaton forms a Cantor set; cellular automaton evolution may be viewed as a continuous mapping on this Cantor set. Quantities such as entropies, dimensions and Lyapunov exponents may then be considered for cellular automata.

In the second approach³, cellular automata are instead considered as information-processing systems (see ref. 15), or parallel-processing computers of simple construction. Information represented by the initial configuration is processed by the evolution of the cellular automaton. The results of this information processing may then be characterized in terms of the types of formal languages generated. (Note that the mechanisms for information processing in natural system appear to be much closer to those in cellular automata than in conventional serial-processing computers: cellular automata may, therefore, provide efficient media for practical simulations of many natural systems.)

Entropies and dimensions

Most cellular automaton rules have the important feature of irreversibility: several different configurations may evolve to a single configuration, and, with time, a contracting subset of all possible configurations appears. Starting from all possible initial configurations, the cellular automaton evolution may generate only special 'organized' configurations, and 'self-organization' may occur.

For class 1 cellular automata, essentially all initial configurations evolve to a single final configuration, analogous to a limit point in a continuous dynamical system. Class 2 cellular automata evolve to limit sets containing essentially only periodic configurations, analogous to limit cycles. Class 3 cellular automata yield chaotic aperiodic limit sets, containing analogues of chaotic or 'strange' attractors.

Entropies and dimensions give a generalized measure of the density of the configurations generated by cellular automaton evolution. The (set) dimension or limiting (topological) entropy for a set of cellular automaton configurations is defined as (compare ref. 14)

$$d^{(x)} = \lim_{X \rightarrow \infty} \frac{1}{X} \log_k N(X) \quad (2)$$

where $N(X)$ gives the number of distinct sequences of X site values that appear. For the set of possible initial configurations, $d^{(x)} = 1$. For a limit set containing only a finite total number of configurations, $d^{(x)} = 0$. For most class 3 cellular automata, $d^{(x)}$ decreases with time, giving, $0 < d^{(x)} < 1$, and suggesting that a fractal subset of all possible configurations occurs.

A dimension or limiting entropy $d^{(t)}$ corresponding to the time series of values of a single site may be defined in analogy with equation (2). (The analogue of equation (2) for a sufficiently wide patch of sites yields a topologically-invariant entropy for the cellular automaton mapping.) $d^{(t)} = 0$ for periodic sets of configurations.

$d^{(x)}$ and $d^{(t)}$ may be modified to account for the probabilities of configurations by defining

$$d_{\mu}^{(x)} = - \lim_{X \rightarrow \infty} \frac{1}{X} \sum_{j=1}^{k^X} p_j \log_k p_j \quad (3)$$

and its analogue, where p_j are probabilities for possible length X sequences. These measure dimensions may be used to delineate the large time behaviour of the different classes of cellular automata:

- (1) $d_{\mu}^{(x)} = d_{\mu}^{(t)} = 0$
- (2) $d_{\mu}^{(x)} > 0$, $d_{\mu}^{(t)} = 0$
- (3) $d_{\mu}^{(x)} > 0$, $d_{\mu}^{(t)} > 0$

As discussed below, dimensions are usually undefined for class 4 cellular automata.

Information propagation

Cellular automata may also be characterized by the stability or predictability of their behaviour under small perturbations in initial configurations. Figure 2 shows differences in patterns generated by cellular automata resulting from a change in a

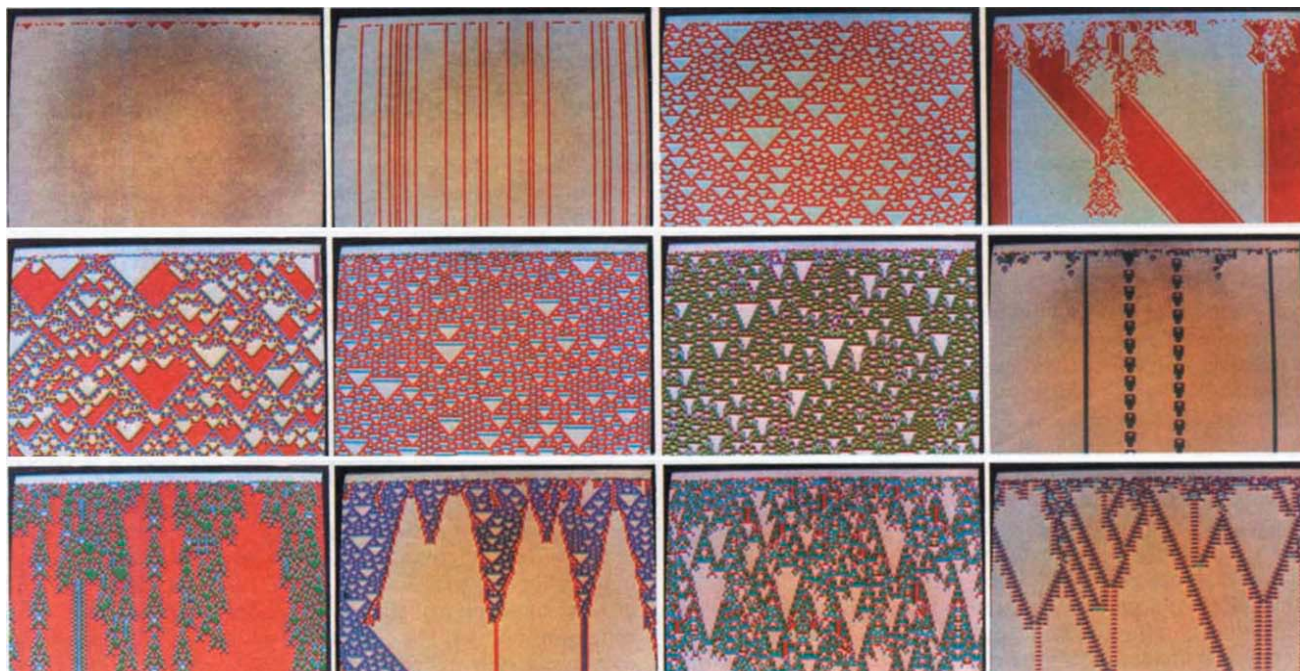


Fig. 3 Evolution of various cellular automata from disordered initial states. In many cases, ordered structure is seen to be generated. The first row of pictures show examples of the four qualitative classes of behaviour found. (The rules shown are the same as in Fig. 1.) The lower two rows show examples of cellular automata with $k=5$ (five possible values for each site) and $r=1$ (nearest neighbour rules). Site values 0 to 4 are represented by white, red, green, blue and yellow squares, respectively. (The rules shown have totalistic codes 10175, 566780, 570090, 580020, 583330, 672900, 5694390, 59123000.) The 'orange' discoloration is a background, not part of the pattern.

Fig. 4 Evolution of multiple phases in cellular automata. Pairs of sites are shown combined: 00 is represented by white, 01 by red, 10 by green and 11 by blue. Alternate time steps are shown. Both rules simulate an additive rule (number 90) under a blocking transformation. In the first rule (number 18), the simulation is attractive: starting from a disordered initial state, the domains grow with time. In the second rule (number 94), the simulation is repulsive: only evolution from a special initial state yields additive rule behaviour; a defect is seen to grow, and attractive simulation of the identity rule takes over.

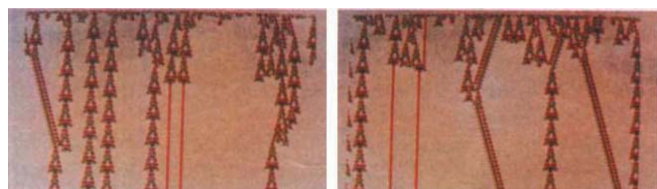
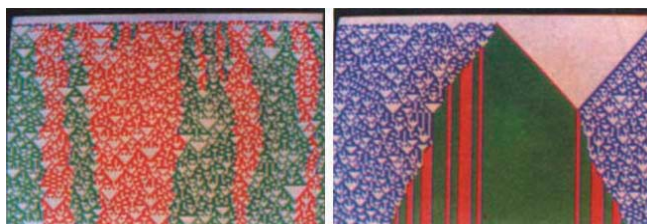


Fig. 5 Examples of the evolution of a typical class 4 cellular automaton from disordered initial states. This and other class 4 cellular automata are conjectured to be capable of arbitrary information processing, or universal computation. The rule shown has $k=3$, $r=1$, and takes the value of a site to be 1 if the sum of the values of the sites in its three-site neighbourhood is 2 or 6, to be 2 if the sum is 3, and to zero otherwise (totalistic code 792).



Fig. 6 Persistent structures generated in the evolution of the class 4 cellular automaton of Fig. 5. The first four structures shown have periods 1, 20, 16 and 12 respectively; the last four structures (and their reflections) propagate: the first has period 32, the next three period 3, and the last period 6. These structures are some of the elements required to support universal computation.

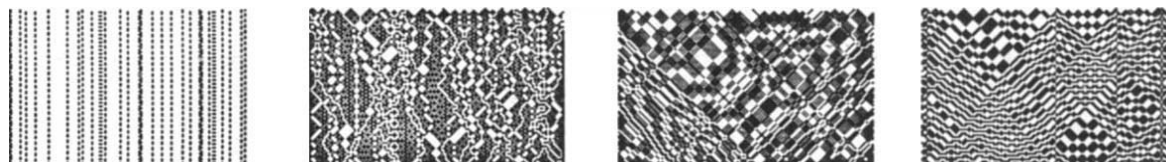


Fig. 7 Evolution of some cellular automata with reversible rules. Each configuration is a unique function of the two previous configurations. (Rule numbers 4, 22, 90 and 126 are shown.) As initial conditions, each site in two successive configurations is chosen to have value 1 with probability 0.1.

single initial site value. Such perturbations have characteristic effects on the four classes of cellular automata:

- (1) no change in final state;
- (2) changes only in a finite region;
- (3) changes over an ever-increasing region;
- (4) irregular changes.

In class 1 and 2 cellular automata, information associated with site values in the initial state propagates only a finite distance; in class 3 cellular automata, it propagates an infinite distance at a fixed speed, while in class 4 cellular automata, it propagates irregularly, but over an infinite range. The speed of information propagation is related to the Lyapunov exponent for the cellular automaton evolution, and measures the degree of sensitivity to initial conditions (see ref. 16). It leads to different degrees of predictability for the outcome of cellular automaton evolution:

- (1) entirely predictable, independent of initial state;
- (2) local behaviour predictable from local initial state;
- (3) behaviour depends on an ever-increasing initial region;
- (4) behaviour effectively unpredictable.

Information propagation is particularly simple for the special class of additive cellular automata (whose local rule function ϕ is linear modulo k), in which patterns generated from arbitrary initial states may be obtained by superposition of patterns generated by evolution of simple initial states containing a single non-zero site. A rather complete algebraic analysis of such cellular automata may be given¹⁷. Most cellular automata are not additive; however, with special initial configurations it is often possible for them to behave just like additive rules. Thus, for example, the evolution of an initial configuration consisting of a sequence of 00 and 01 digrams under one rule may be identical to the evolution of the corresponding 'blocked' configuration consisting of 0 and 1 under another rule. In this way, one rule may simulate another under a blocking transformation (analogous to a renormalization group transformation). Evolution from an arbitrary initial state may be attracted to (or repelled from) the special set of configurations for which such a simulation occurs. Often several phases exist, corresponding to different blocking transformations: sometimes phase boundaries move at constant speed, and one phase rapidly takes over; in other cases, phase boundaries execute random walks, annihilating in pairs, and leading to a slow increase in the average domain size, as illustrated in Fig. 4. Many rules appear to follow attractive simulation paths to additive rules, which correspond to fixed points of blocking transformations, and thus exhibit self similarity. The behaviour of many rules at large times, and on large spatial scales, is therefore determined by the behaviour of additive rules.

Thermodynamics

Decreases with time in the spatial entropies and dimensions of equations (2) and (3) signal irreversibility in cellular automaton evolution. Some cellular automaton rules are, however, reversible, so that each and every configuration has a unique predecessor in the evolution, and the spatial entropy and dimension of equations (2) and (3) remain constant with time. Figure 7 shows some examples of the evolution of such rules, constructed by adding a term $-a_i^{(t-1)}$ to equation (1) (ref. 20 and E. Fredkin, personal communication). Again, there are analogues of the

four classes of behaviour seen in Fig. 3, distinguished by the range and speed of information propagation.

Conventional thermodynamics gives a general description of systems whose microscopic evolution is reversible; it may, therefore, be applied to reversible cellular automata such as those of Fig. 4. As usual, the 'fine-grained' entropy for sets (ensembles) of configurations, computed as in equation (3) with perfect knowledge of each site value, remains constant in time. The 'coarse-grained' entropy for configurations is, nevertheless, almost always non-decreasing with time, as required by the second law of thermodynamics. Coarse graining emulates the imprecision of practical measurements, and may be implemented by applying almost any contractive mapping to the configurations (a few iterations of an irreversible cellular automaton rule suffice). For example, coarse-grained entropy might be computed by applying equation (3) to every fifth site value. In an ensemble with low coarse-grained entropy, the values of every fifth site would be highly constrained, but arbitrary values for the intervening sites would be allowed. Then in the evolution of a class 3 or 4 cellular automaton the disorder of the intervening site values would 'mix' with the fifth-site values, and the coarse-grained entropy would tend towards its maximum value. Signs of self-organization in such systems must be sought in temporal correlations, often manifest in 'fluctuations' or metastable 'pockets' of order.

While all fundamental physical laws appear to be reversible, macroscopic systems often behave irreversibly, and are appropriately described by irreversible laws. Thus, for example, although the microscopic molecular dynamics of fluids is reversible, the relevant macroscopic velocity field obeys the irreversible Navier-Stokes equations. Conventional thermodynamics does not apply to such intrinsically irreversible systems: new general principles must be found. Thus, for cellular automata with irreversible evolution rules, coarse-grained entropy typically increases for a short time, but then decreases to follow the fine-grained entropy. Measures of the structure generated by self-organization in the large time limit are usually affected very little by coarse graining.

Formal language theory

Quantities such as entropy and dimension, suggested by information theory, give only rough characterizations of cellular automaton behaviour. Computation theory suggests more complete descriptions of self-organization in cellular automata (and other systems). Sets of cellular automaton configurations may be viewed as formal languages, consisting of sequences of symbols (site values) forming words according to definite grammatical rules.

The set of all possible initial configurations corresponds to a trivial formal language. The set of configurations obtained after any finite number of time steps are found to form a regular language³. The words in a regular language correspond to the possible paths through a finite graph representing a finite state machine. It can be shown that a unique smallest finite graph reproduces any given regular language (see ref. 15). Examples of such graphs are shown in Fig. 8. These graphs give complete specifications for sets of cellular automaton configurations (ignoring probabilities). The number of nodes Ξ in the smallest graph corresponding to a particular set of configurations may

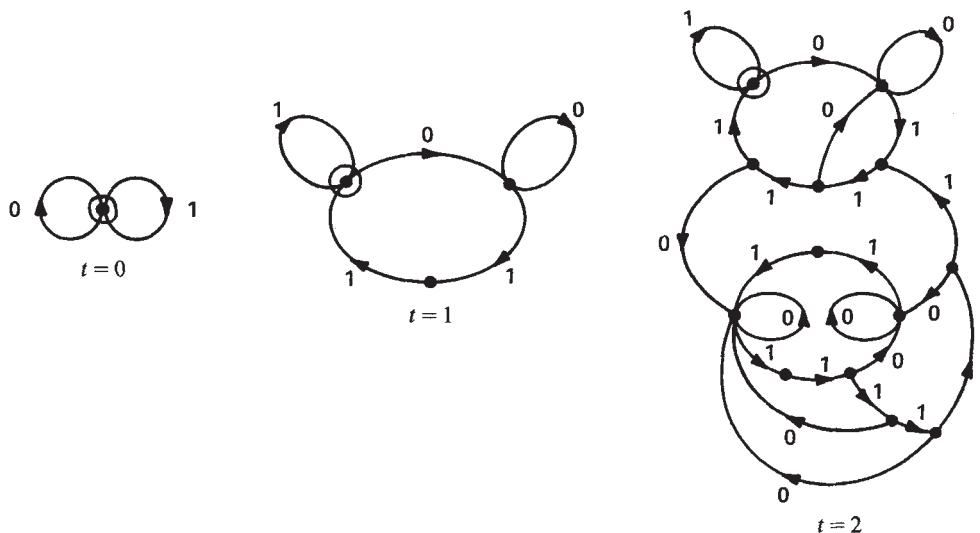


Fig. 8 Graphs representing the sets of configurations generated in the first few time steps of evolution according to a typical class 3 cellular automaton rule ($k=2$, $r=1$, rule number 126). Possible configurations correspond to possible paths through the graphs, beginning at the encircled node. At $t=0$, all possible configurations are allowed. With time, a contracting subset of configurations are generated. (After one time step, for example, no configuration containing the sequence of site value 101 can appear.) At each time step, the complete set of possible configurations forms a regular formal language: the graph gives a minimal complete specification of it. The number of nodes in the graph gives a measure of the complexity Ξ of the set, viewed as a regular language. As for other class 3 cellular automata, the complexity of the sets grows rapidly with time; for $t=3$, $\Xi=107$, and $t=4$, $\Xi=2,867$.

be defined as the 'regular language complexity' of the set. It specifies the size of the minimal description of the set in terms of regular languages. Larger Ξ correspond to more complicated sets. (Note that the topological entropy of a set is given by the logarithm of the algebraic integer obtained as the largest root of the characteristic polynomial for the incidence matrix of the corresponding graph. The characteristic polynomials for the graphs in Fig. 7 are $2-\lambda$ ($\lambda_{\max}=2$), $1-\lambda+2\lambda^2-\lambda^3$ ($\lambda_{\max}\approx 1.755$) and $-1+\lambda-\lambda^2+2\lambda^3-4\lambda^4+\lambda^5+3\lambda^6-5\lambda^7+3\lambda^8-3\lambda^9+5\lambda^{10}-6\lambda^{11}+4\lambda^{12}-\lambda^{13}$ ($\lambda_{\max}\approx 1.732$), respectively.)

The regular language complexity Ξ for sets generated by cellular automaton evolution almost always seems to be non-decreasing with time. Increasing Ξ signals increasing self-organization. Ξ may thus represent a fundamental property of self-organizing systems, complementary to entropy. It may, in principle, be extracted from experimental data.

Cellular automata that exhibit only class 1 and 2 behaviour always appear to yield sets that correspond to regular languages in the large time limit. Class 3 and 4 behaviour typically gives rise, however, to a rapid increase of Ξ with time, presumably leading to limiting sets not described by regular languages.

Formal languages are recognized or generated by idealized computers with a 'central processing unit' containing a fixed finite number of internal states, together with a 'memory'. Four types of formal languages are conventionally identified, corresponding to four types of computer:

- Regular languages: no memory required.
- Context-free languages: memory arranged as a last-in, first-out stack.
- Context-sensitive languages: memory as large as input word required.
- Unrestricted languages: arbitrarily large memory required (general Turing machine).

Examples are known of cellular automata whose limiting sets correspond to all four types of language (L. Hurd, in preparation). Arguments can be given that the limit sets for class 3 cellular automata typically form context-sensitive languages, while those for class 4 cellular automata correspond to unrestricted languages. (Note that while a minimal specification for any regular language may always be found, there is no finite procedure to obtain a minimal form for more complicated formal languages: no generalization of the regular language complexity Ξ may thus be given.)

Computation theory

While dynamical systems theory concepts suffice to define class 1, 2 and 3 cellular automata, computation theory is apparently required for class 4 cellular automata. Examples of the evolution of a typical class 4 cellular automaton are shown in Fig. 5. Varied and complicated behaviour, involving many different time scales is evident. Persistent structures are often generated; the smallest few are illustrated in Fig. 6, and are seen to allow both storage and transmission of information. It seems that the structures supported by this and other class 4 cellular automata rule may be combined to implement arbitrary information processing operations. Class 4 cellular automata would then be capable of universal computation: with particular initial states, their evolution could implement any finite algorithm. (Universal computation has been proved for a $k=18$, $r=1$ rule²², and for two-dimensional cellular automata such as the 'Game of Life'^{22,23}.) A few per cent of cellular automaton rules with $k>2$ or $r>1$ are found to exhibit class 4 behaviour: all these would then, in fact, be capable of arbitrarily complicated behaviour. This capability precludes a smooth infinite size limit for entropy or other quantities: as the size of cellular automaton considered increases, more and more complicated phenomena may appear.

Cellular automaton evolution may be viewed as a computation. Effective prediction of the outcome of cellular automaton evolution requires a short-cut that allows a more efficient computation than the evolution itself. For class 1 and 2 cellular automata, such short cuts are clearly possible: simple computations suffice to predict their complete future. The computational capabilities of class 3 and 4 cellular automata may, however, be sufficiently great that, in general, they allow no short-cuts. The only effective way to determine their evolution from a given initial state would then be by explicit observation or simulation: no finite formulae for their general behaviour could be given. (If class 4 cellular automata are indeed capable of universal computation, then the variety of their possible behaviour would preclude general prediction, and make explicit observation or simulation necessary.) Their infinite time limiting behaviour could then not, in general, be determined by any finite computational process, and many of their limiting properties would be formally undecidable. Thus, for example, the 'halting problem' of determining whether a class 4 cellular automaton with a given finite initial configuration ever evolves to the null configuration would be undecidable. An explicit simulation could determine

only whether halting occurred before some fixed time, and not whether it occurred after an arbitrarily long time.

For class 4 cellular automata, the outcome of evolution from almost all initial configurations can probably be determined only by explicit simulation, while for class 3 cellular automata this is the case for only a small fraction of initial states. Nevertheless, this possibility suggests that the occurrence of particular site value sequences in the infinite time limit is in general undecidable. The large time limit of the entropy for class 3 and 4 cellular automata would then, in general, be non-computable: bounds on it could be given, but there could be no finite procedure to compute it to arbitrary precision. (This would be the case if the limit sets for class 3 and 4 cellular automata formed at least context-sensitive languages.)

While the occurrence of a particular length n site value sequence in the infinite time limit may be undecidable, its occurrence after any finite time t can, in principle, be determined by considering all length $n_0 = n + 2rt$ initial sequences that could evolve to it. For increasing n or t this procedure would, neverthe-

less, involve exponentially-growing computational resources, so that it would rapidly become computationally intractable. It seems likely that the identification of possible sequences generated by class 3 and 4 cellular automata is, in general, an NP-complete problem (see ref. 15). It can, therefore, presumably not be solved in any time polynomial in n or t , and essentially requires explicit simulation of all possibilities.

Undecidability and intractability are common in problems of mathematics and computation. They may well afflict all but the simplest cellular automata. One may speculate that they are widespread in natural systems, perhaps occurring almost whenever nonlinearity is present. No simple formulae for the behaviour of many natural systems could then be given; the consequences of their evolution could be found effectively only by direct simulation or observation.

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ARTICLES

Orientation of *in situ* stresses in the oceanic crust

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Two in situ measurements of principal stress directions have been made in DSDP Holes 504B, south of the Costa Rica Rift on the Nazca plate, and 597C, west of the East Pacific Rise on the Pacific plate. In both cases, the orientations of in situ principal stresses determined from borehole breakouts are consistent with the stress directions inferred from intraplate earthquakes located near the sites.

WELLBORE breakouts are intervals of borehole elongation which are caused by preferential spalling in a zone where the circumferential compressive stress is greatest¹, and in which the average azimuth of the long dimension is consistent in a given well or field^{2,3}. Stress-induced wellbore breakouts form at an azimuth perpendicular to the maximum horizontal principal stress direction¹. Wellbore breakouts have been reported in wells from several parts of North America²⁻¹⁵ but there have been problems in their identification (see ref. 16). There now seems to be ample evidence that breakouts can be used as a reliable indicator of the orientation of the horizontal principal stresses. Here, we use specially processed borehole televiwer data to study the detailed shape of breakouts (see ref. 1).

Borehole televiwer reflectivity records have previously been used in the study of fractures intersecting wellbores at depth (see ref. 17), for identifying lithostratigraphical features in deep ocean boreholes (see refs 18, 19), and in identifying zones of borehole breakout^{1,20,21}.

Experimental data

Evidence of the orientation of the contemporary *in situ* stress field can be found at two DSDP (Deep Sea Drilling Project) sites in the oceanic crust (Fig. 1). DSDP Site 504 is located 200 km due south of the Costa Rica Rift and about 350 km north-west of the Peru-Chile Trench. DSDP Site 597 is located about 1,800 km west of the East Pacific Rise and 2,000 km east of Tahiti.

During DSDP Leg 83, the DV *Glomar Challenger* completed drilling Hole 504B to a depth of 1,350 m, 1,000 m of which was into basement²² (Fig. 1). The upper 100 m of basement¹⁹ is composed mainly of pillow basalts and thin flow units of seismic layer 2A. The next 550 m of pillows, breccias and flows is a site of intense fracturing, and contains extensive secondary alteration; it is thought to be seismic layer 2B. The bottom 350 m of the hole is composed mainly of massive, 'welded' units, or sheeted dykes, and is the upper part of seismic layer 2C.

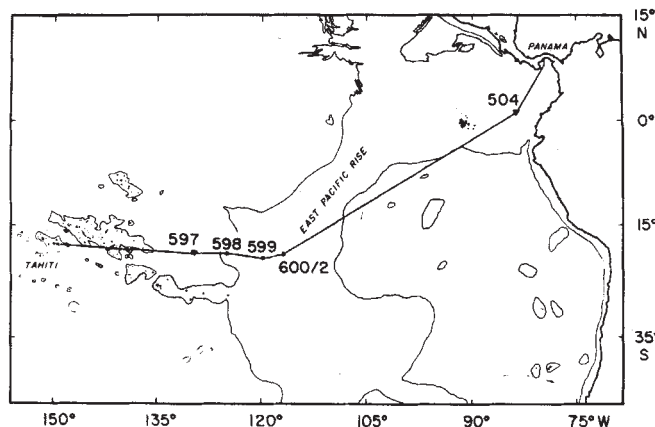


Fig. 1 Location of DSDP Sites 504 and 597 along ship track for DSDP Leg 92.

Borehole televiwer (BHTV) images run in Hole 504B suggested the presence of breakouts at many intervals down the hole. Our breakout analysis of the BHTV records was confined to the lower 350 m of the hole, because the borehole walls in the lower part of the hole are relatively smooth (apart from the breakout intervals), in comparison to the washed out, pitted and broken nature of the wellbore in the upper zones of pillows and breccias. For analysis of the BHTV records, cross-sectional travel-time images were produced every few centimetres in breakout zones in the lower 350 m of the hole. These form 360° caliper images of the wellbore. The criteria which were used to confirm the breakouts included: (1) the wellbore should have two bands of spalling, separated by $180^\circ \pm 20^\circ$; and (2) evidence of the same cross-sectional outline and orientation should persist over several (>4) consecutive rotations of the tool's acoustic transducer, indicating breakout continuity of several centimetres for each measurement.

Figure 2b shows a reflectivity record of a 5 m-long interval from Hole 504B which is oriented with respect to magnetic north. Hard, smooth surfaces produce white zones (high-amplitude reflectance), and fractures, voids and soft material, which would absorb much of the signal, produce dark zones (low-

amplitude reflectance). The vertical, dark bands seen at azimuths of about 120° and 300° are breakout zones. Figure 2c shows a cross-sectional, 360° caliper view of the breakout at a depth of 944 m into basement which was made by plotting the travel time of the reflected acoustic pulse as a function of azimuth.

In all, 65 measurements of orientations of breakouts which conformed to the above listed criteria were made in the records obtained in the lower 350 m of Hole 504B. Figure 3b shows examples of breakout cross-sections at other depths through this depth interval. The azimuths (with respect to magnetic north) of the midpoints of each breakout zone are plotted against depth in Fig. 3a. The mean azimuths are $N112E \pm 16^\circ$ ($\pm$ s.d.) and $N296E \pm 16^\circ$, with a mean separation of $184^\circ \pm 11^\circ$. This indicates an average maximum horizontal compressional stress oriented at $N24E$ magnetic, which, when corrected for magnetic declination (4.5° east), results in a maximum horizontal stress direction of $N20E \pm 16^\circ$ at the site.

During DSDP Leg 92, as part of a hydrogeology investigation (Fig. 1), a series of holes was drilled at sites which form a west-east transect across the western flank of the East Pacific Rise, at 19° South. Hole 597C, a re-entry hole, was drilled 91 m into the 28.5-Myr-old crust, generated at the now-extinct but fast-spreading Galapagos Rise. Massive flows appear to comprise the entire sequence. This is supported by both the BHTV amplitude records and the core recovery which ranged up to 94%. Only one small fragment which might possibly be a pillow margin was recovered²³. The caliper logs indicate that the borehole has few abrupt changes in diameter, and the BHTV amplitude records show the wellbore as having a generally uniform and solid appearance, with few zones of low reflectivity.

In some intervals, zones of borehole breakout oriented NNE-SSW were identified. Because of the otherwise smooth and solid character of the wellbore, the presence of breakouts is readily recognized in travel-time processing of the BHTV records (Fig. 3d). In all, 18 measurements of breakout orientations were made in the BHTV records from the upper 50 m of basement in Hole 597C (Fig. 3c, d). Unlike the measurements from Hole 504B, each of these measurements was made using data from only two rotations of the tool because cold formation temperatures slowed the speed of tool rotation. The azimuths (with respect to magnetic north) of the midpoints of each breakout zone are plotted against depth in Fig. 3c. The mean azimuths are $N30E$

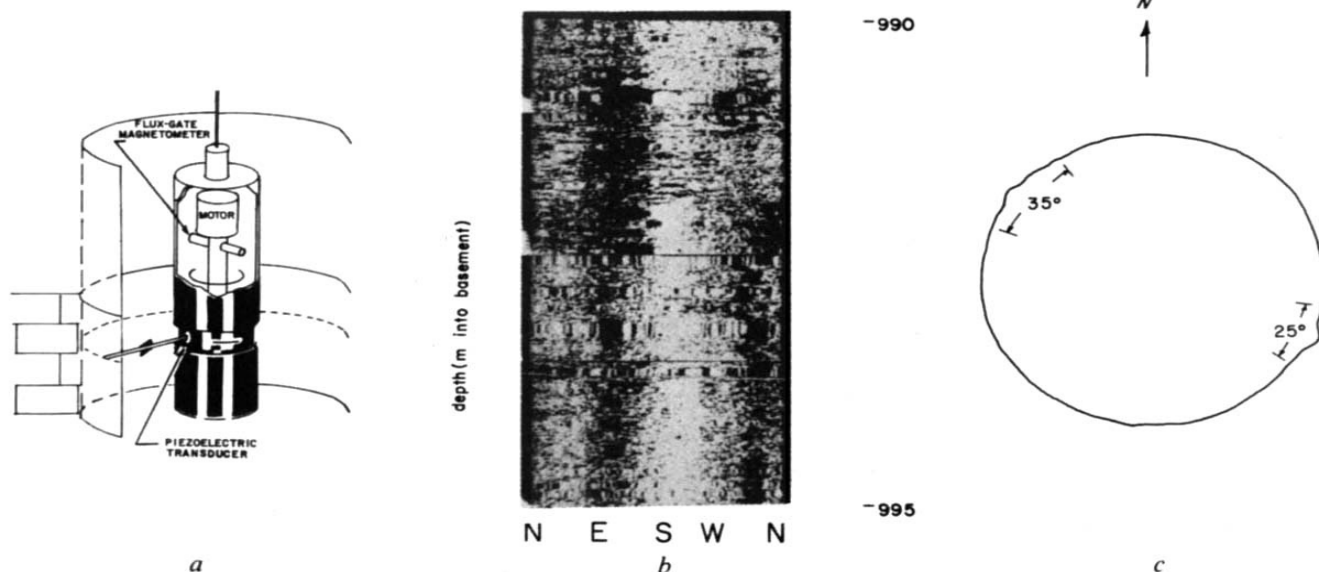


Fig. 2 a, Schematic of the borehole televiwer. The televiwer contains a rotating piezoelectric transducer which emits and receives an ultrasonic pulse 600 times a revolution as it rotates three times per s^{33} . The amplitude of the reflected acoustic pulse can be plotted on a three-axis oscilloscope as a function of beam azimuth and vertical position in the hole, producing an image of wellbore reflectivity, or smoothness. The travel time of the reflected acoustic pulse can be plotted as a function of beam azimuth, which results in a detailed cross-sectional image of the wellbore. A fluxgate magnetometer triggers the scope trace at magnetic north so that the orientation of observed features can be determined. b, Amplitude, or plan-view borehole televiwer record of 5 m of Hole 504B. Width across image is about 1 m. Dark vertical bands oriented ESE-WNW are breakout zones. c, Cross-section of Hole 504B at a depth of 944 m into basement, showing breakout.

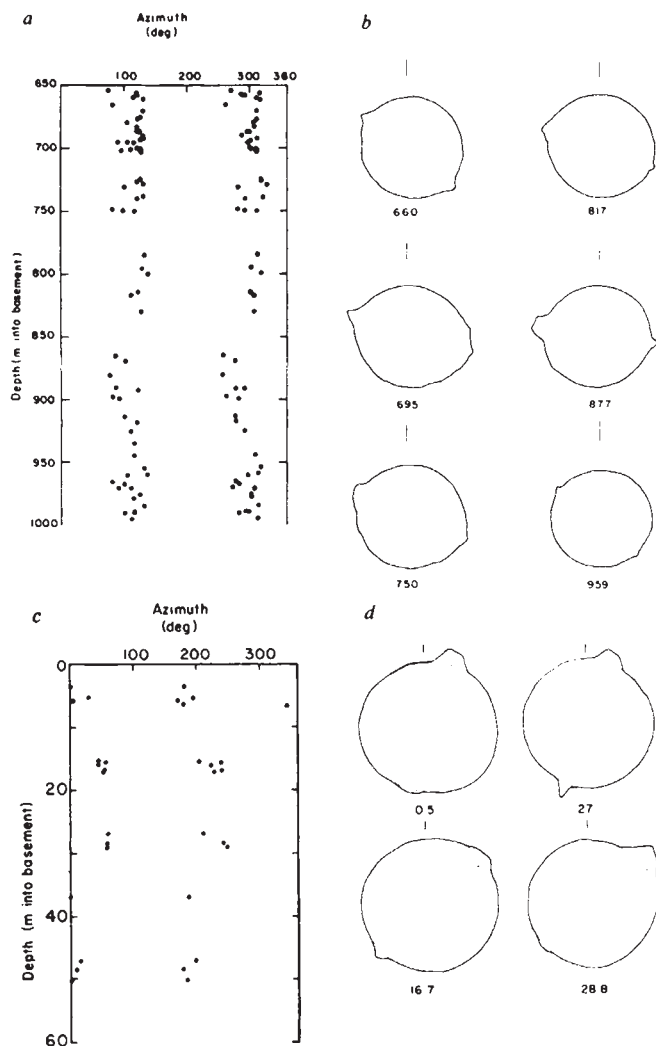


Fig. 3 Azimuths (with respect to magnetic north) of the mid-points of borehole breakouts plotted against depth in *a*, Hole 504B; *c*, Hole 597C. Mean azimuths are $N112E$ and $N296E \pm 16^\circ$ (*a*) and $N30E$ and $N208E \pm 25^\circ$ (*c*). Examples of breakout cross-sections in *b*, Hole 504B; *d*, Hole 597C. Vertical lines indicate true north. Numbers indicate depth in metres into basement.

and $N208E \pm 25^\circ$, with a mean separation of 178° . This indicates an average maximum horizontal compressional stress direction of $N121E$ magnetic, which, when corrected for magnetic declination results in a maximum horizontal stress oriented $N110E \pm 25^\circ$ at the site.

Discussion

DSDP Hole 504B is located 200 km due south of the east-west-trending Costa Rica Rift and about 350 km NW of the NE-SW-trending Peru-Chile Trench. Ridge push from the Costa Rica Rift should produce a southward compression at the site. The net slab pull, or the sum of driving and resisting slab forces, should produce southeastward extensional stresses at the site (Fig. 4).

As shown in Fig. 4, the observed maximum principal horizontal stress direction is oriented almost parallel to the north-south compression expected from a ridge-push force from the Costa Rica Rift. This would seem to suggest the dominance of the ridge-push force at this site. However, the slab pull force associated with subduction of the Nazca plate along the Peru-Chile Trench is expected to generate east-southeast-oriented extensional stresses. Because the observed direction of least principal stress is $N110E$, it is nearly parallel to the predicted direction

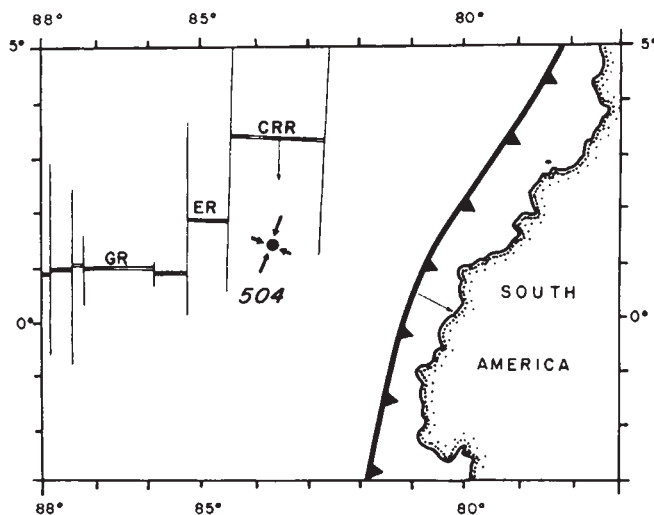


Fig. 4 Stresses near Site 504. Heavy arrows indicate the principal horizontal compressive stress directions determined from breakout directions at Hole 504B. Light arrows show the direction of ridge-push force from the Costa Rica Rift and the direction of the slab-pull force from the Peru-Chile Trench. Double lines indicate the ridge axis of the Cocos-Nazca spreading system and hatched line is the Peru-Chile Trench³². CRR, Costa Rica Rift; ER, Ecuador Rift; GR, Galapagos Rift.

of trench pull. Thus, it is not clear from the orientation of the principal horizontal stresses at Hole 504B whether ridge push, or trench pull, is the dominant mechanism controlling the *in situ* stress field. In any event, this direction is consistent with focal mechanism solutions of earthquakes located near the site, which exhibit a dominant NNE-SSW P-axis orientation²⁴.

DSDP Hole 597C is located $\sim 1,800$ km west of the East Pacific Rise (Fig. 1). Ridge push from the East Pacific Rise should produce west-northwest-oriented compression at the site. As can be seen in Fig. 5, the maximum horizontal compression at Site 597C is parallel to both the relative and absolute plate motion vectors for the Pacific plate, both of which are consistent with an interpretation of a dominant ridge-push force acting at the site.

The shaded areas in Fig. 5 indicate the local range of uncertainty in identifying the Okal and Bergeal 'J' line²⁵ which separates lithosphere generated at the Galapagos Rise before the Miocene ridge jump from lithosphere generated at the East Pacific Rise. It is a line of age discontinuity and may represent a zone of weakness in the plate. Because the ridge jump was accompanied by a change in the azimuth of spreading, the J line separates lithosphere with differing tectonic orientations. Fracture zones trend $N250E$ in the older lithosphere and $N290E$ in the younger lithosphere.

The anomalous intraplate seismicity in this area graphically demonstrates a change in the consistency of stress orientations inferred from intraplate earthquakes located in crust on either side of the J line. Focal mechanism solutions of several intraplate earthquakes located in the general vicinity of Site 597 are shown in Fig. 5 (refs 24, 26-29).

LI2, GB4 and GB5 correspond to Okal *et al.*'s²⁶ regions A, B and C, respectively. Each of these clusters has horizontal dimensions of ≤ 50 km. All solutions indicate focal depths of ≤ 5 km. A seismic network in French Polynesia has provided a detailed account of the seismicity in these regions over the past several years²⁶.

At LI2, activity is characterized by swarms, mostly with strike-slip solutions with some normal component. The P axes are oriented WNW-ESE which is nearly parallel to both the current relative and absolute plate motion vectors. There is no evidence of related local bathymetric features.

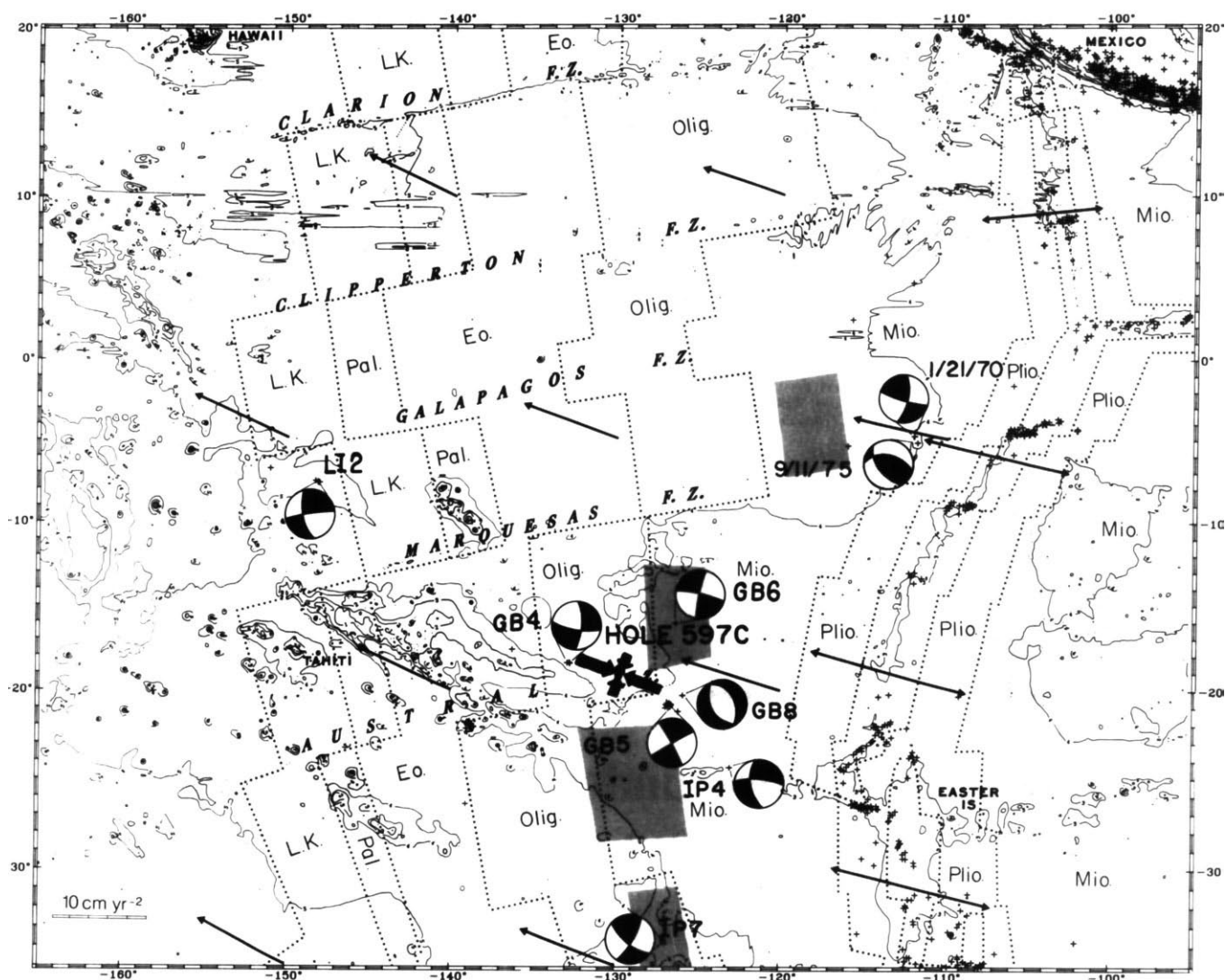


Fig. 5 Stresses near Site 597C. Heavy arrows indicate the principal horizontal stress directions determined from breakout directions at Hole 597C. Light single-headed arrows indicate the absolute Pacific plate motion, and light double-headed arrows show relative Pacific-Nazca plate motion³⁴. The trend of the East Pacific Rise is shown by crosses which mark the locations of earthquakes with $m_b > 4.5$ mag and recorded by more than 10 stations by the International Seismological Centre between 1963 and 1979³⁵. Focal mechanism solutions are discussed in the text.

At GB4, activity occurred as discrete events through time, again mostly characterized by strike-slip solutions with some normal component. There were 12 events with body-wave magnitudes of up to 5.5 during 15 yr.

At GB5, there was a 3-yr swarm of intense activity from 1976 to 1979, with over 96 events—mostly characterized by strike-slip solutions, but unlike at LI2 or GB4, with some thrust component. This swarm was followed by a 3-yr period of quiescence when there was no activity with magnitude > 4 . In July 1983, a main-shock–aftershock pair of events occurred only 65 km away at GB8, with a purely normal solution, at depths of 15–20 km. There is no evidence of related local bathymetric features in the area.

IP4 is the site of one of the largest normal faulting events known in young oceanic lithosphere, with a body-wave magnitude of 6.8. Its epicentre lies north-west of a major seamount to which this activity may be related. IP7 is located in the range of uncertainty of the J line.

The focal mechanism solutions of earthquakes located in older crust (LI2 and GB4) indicate P axes oriented WNW–ESE, which are consistent with the interpretation of a dominant ridge-push force acting on the plate. However, focal mechanism solutions of events located at sites near the J line or in younger crust show more diverse orientations of P axes. At GB5 and GB8,

events occurred with normal, thrust and strike-slip mechanisms, within 3 yr and 65 km.

Our Site 597 is located in crust just older than the J line and indicates a dominant stress orientation which is consistent with the stress regime inferred by the intraplate events in older crust. This is particularly satisfying because our measurement was made only in the upper 50 m of the oceanic crust.

Conclusions

Most events far from plate boundaries indicate compressive stresses which are thought to result from the gravitational force due to lithospheric thickening with age, or ridge-push force. However, both compressional and extensional events occur in young lithosphere. Various authors^{29–31} have proposed ages of from 10 to 35 Myr for the transition between the near-ridge tectonic regime which may be dominated by local forces and the stress regime in stable plate interiors characterized by horizontal compression. Site 597 seems to be located very close to this transition zone west of the East Pacific Rise.

To date, two *in situ* measurements of principal stress orientations have been made in DSDP holes: at Hole 504B, south of the Costa Rica Rift on the Nazca plate and at Hole 597C, west of the East Pacific Rise on the Pacific plate. In both cases, the

orientation of *in situ* principal stresses determined from borehole breakouts have been consistent with the stress directions inferred from intraplate earthquakes near the sites. The important point of this work is the demonstration that stress orientations can be reliably determined from borehole breakouts in the oceanic crust. With the advent of a new phase of deep ocean drilling, we are confident that these measurements, routinely made in boreholes at locations oriented with respect to major plate boundaries, will allow us to define more clearly the state of stress in the upper oceanic crust and the relationships between the driving forces of plate tectonics.

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Mantle upflow under North America and plate dynamics

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In the development of plate dynamics there are two fundamental questions. First, do the plates slide over a passive asthenosphere, or are they driven by a convecting upper mantle? Second, are all ocean ridges passive, pull-apart plate margins, or do some lie above upcurrents in the mantle and in that sense become active margins? The combination of stress orientations in the crust of North America with other geophysical and geological information favours an active mantle interpretation, both for the North American plate and for the mantle upflow which lay beneath the East Pacific Rise in Tertiary times and which now lies beneath the western United States.

THE fundamental hypothesis of plate tectonics, that the Earth has a strong outer shell, or lithosphere, made up of a small number of quasi-rigid plates, is now well established. Earthquakes occur mainly along, and are used to locate, plate boundaries. Linear marine magnetic anomalies and earthquake mechanisms have been used to construct a detailed picture of the motions of the plates. The next step is to develop a similarly satisfactory account of the forces driving the plates. In this development of plate dynamics, an important question is whether the plates slide over a passive weak layer, or asthenosphere, under gravitational forces acting only on the plates themselves, or are driven by convection in the mantle through the asthenosphere as a coupling layer. In the latter case, one would expect to find active ocean ridges driven by upcurrents in the mantle, probably with rapid spreading rates; and probably also passive ridges where slower spreading is driven only by gravity acting on the plates. In the past 30 Myr, the boundary between the North American plate and adjacent plates under the Pacific Ocean has undergone a complex evolution. This article brings together a wide range of geological, geophysical and geochemical information to show that there is now an

upward flow of mantle material under the western United States. The implication is that active convection in the mantle is driving the Pacific plate and maintaining compressive stress, in a NE-SW direction, in the North American plate.

Two well-known reconstructions^{1,2} of the Cenozoic tectonic evolution of the Pacific–North American plate boundary started with a continuous subduction along the western margin of the continent, the Farallon Plate between that subduction and the East Pacific Rise (EPR) with its associated transform faults, and the Pacific Plate west of the EPR. After the first contact of the EPR and its transform faults with the subduction, some 30 Myr BP, triple junctions migrated to the south-east and north-west and replaced the subduction with the San Andreas transform fault system. In this process, the oceanic Farallon Plate disappeared, except its remnants the Gorda, Juan de Fuca and Explorer plates north of 40° N and the Cocos plate south of 23° N, all of which are still being subducted under North America.

For the present purpose I adopt this widely accepted model of the sequence of plate interactions over the past 50 Myr. If (as Atwater² assumed) the EPR had been a passive, pull-apart

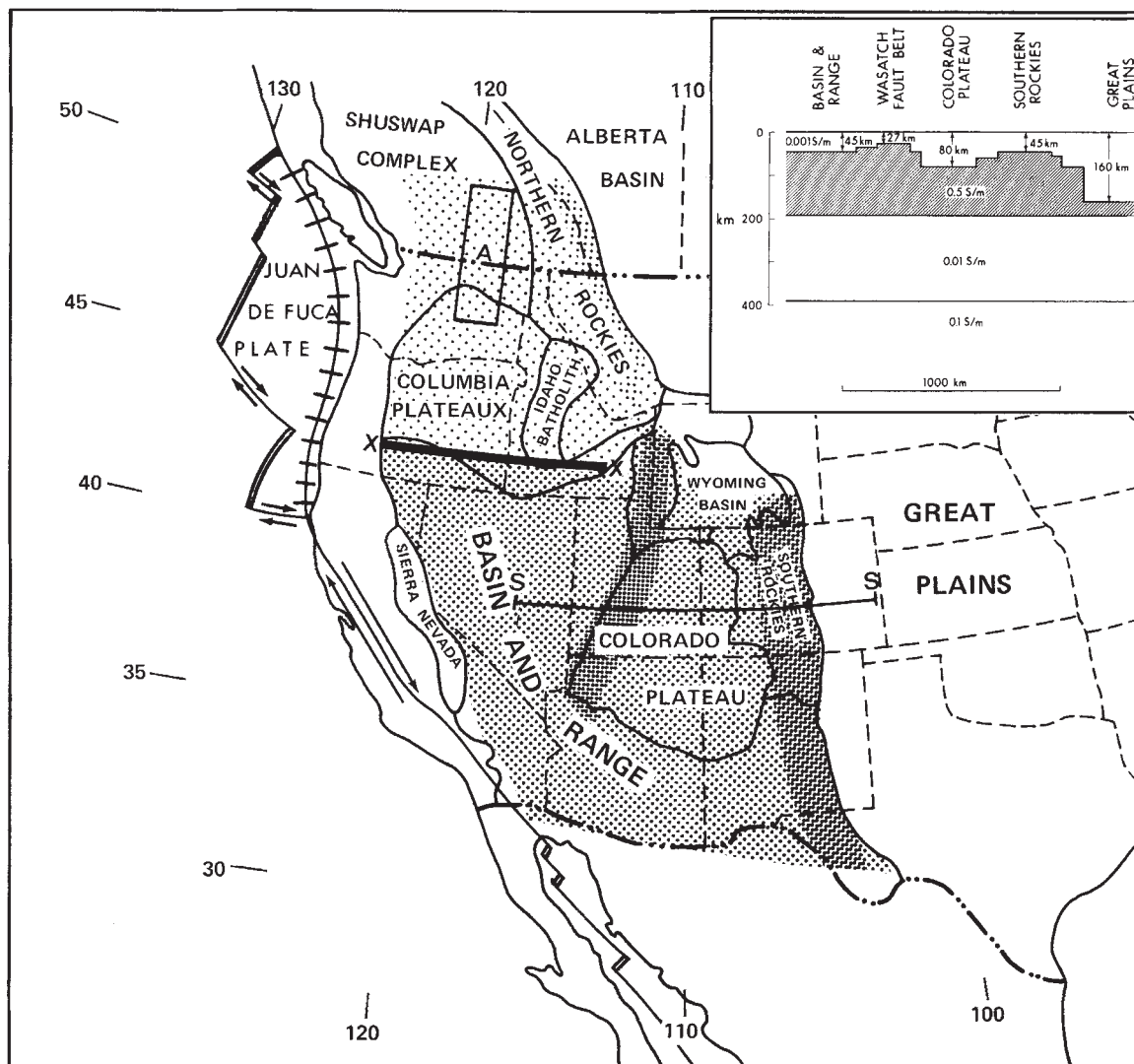


Fig. 1 The density of strippling on the map indicates variations of electrical conductance of an upper mantle layer. It is also believed to show partial melting. The section shows a model of conductive structure²⁶ along the east-west section SS. Major tectonic provinces are indicated.

margin, its replacement with the San Andreas Fault would not imply any marked thermal aftereffects within the continent. But if the EPR of Cenozoic times lay above a mantle upflow, such an upflow might now be present under the western US. Either possibility is consistent with earlier models^{1,2} of the kinematics of the plates.

High temperatures and partial melting

There is abundant geophysical and geological evidence that temperatures in the upper mantle west of the undisturbed North American craton are higher than those beneath it. The boundary of the high-temperature régime lies at the eastern front of the Rocky Mountains^{3,4}. West of this boundary the cratonic rocks of the Southern Rockies and Colorado Plateau (Fig. 1) have been uplifted in the Laramide orogeny⁵, largely as a result of heating of the underlying upper mantle. The topographic uplift itself is highly significant as an indicator of the high-temperature region. Such features as the Colorado Plateau and the Grand Canyon show uplift of 3–4 km, and many mountains in the western US are peneplaned in this range of elevation. The igneous petrology of the region is even more significant. West and south of the Colorado Plateau the Basin and Range Province, and the Columbia Plateaux further north, are dominated by volcanic rocks of two types. Calc-alkaline andesitic volcanics were extruded in late Cretaceous and early Tertiary times, up

to about 30 Myr BP (ref. 6). This epoch corresponds to the subduction regime in Atwater's² scheme, before the most easterly ridge segment reached the trench. The earlier, andesitic volcanism can, therefore, be ascribed to ascent of melt from the downgoing plate on the model of Ringwood and Green⁷, as shown in detail by Christiansen and Lipman⁸. Uplift of the Southern Rockies and Colorado Plateau probably occurred largely at that stage⁵. After 25 Myr BP basaltic volcanism dominated the Basin and Range Province and Columbia Plateaux⁸. The basalts were extruded in great volume in an extensional stress field with most faults trending north–south. All of these observations receive a clear explanation if a mantle upflow, formerly feeding an active ridge at the EPR, lay under the Basin and Range Province after replacement of the ridge and trench with the San Andreas fault system. This is the explanation long held by J. Tuzo Wilson (ref. 9 and personal communications) and myself. Others^{8,10} have preferred to regard the extensional fractures and basaltic extrusions as the result of oblique shearing transmitted from the San Andreas faults. This is probably the best account possible if one assumes that the EPR of early Tertiary times was a passive, pull-apart margin.

Regional variations of heat flow from the mantle confirm the thermal structure indicated by the volcanic rocks^{3,4}. Seismology and electromagnetic geophysics reinforce the regional variations and add information in the depth dimension. For brevity the continent east of the Rocky Mountains is termed the 'craton',

the region west of the craton the 'western region' within which lies the Basin and Range Province. Teleseismic P and S waves arriving vertically are delayed in the western region compared with the craton¹¹⁻¹³ in such a way as to show that the rigidity modulus is reduced much more than the bulk modulus under the western region¹⁴, which would be the case if partial melting were present in the upper mantle there. Seismic refraction shows that the uppermost mantle, just under the crust, transmits P_n waves with lower velocities in the western region than in the craton, with the lowest velocities in the Basin and Range Province. Results from long seismic refraction lines show the presence of a marked low-velocity layer for P waves in the upper mantle under the western region¹⁵⁻¹⁷, but not under the craton. In a representative recent model¹⁸, the low-velocity layer lies in the depth range 65–200 km. Recent studies of attenuation of P and S teleseismic waves¹⁹ have confirmed large variations under the US, with greatest attenuation under the western region including, but not confined to, the Basin and Range Province, and lowest attenuation under the north central shield of the craton. Seismological studies, therefore, define a region in which the elastic body wave velocities are reduced, and their amplitudes attenuated through anelastic response, in a layer in the upper mantle under the western region, in general, and the Basin and Range Province, in particular.

Observations of natural time variations of the geomagnetic field, with two-dimensional arrays of recording magnetometers, can be used to discover and map rock structures of anomalously high electrical conductivity²⁰. Figure 1 summarizes the results of the first three such studies with arrays of magnetometers²¹⁻²⁴. The density of stippling on the map gives a qualitative indication of the conductance (=conductivity × thickness) of the anomalously conductive layer. This is confined to the region west of the craton, with high conductance under the Basin and Range Province and the Colorado Plateau, and still higher conductance under the Southern Rockies and Rio Grande Rift (where Schmucker²⁵ first found a highly-conductive ridge in the upper mantle) and under the Wasatch Front mountains of Utah. The magnetovariation fields alone give poor discrimination in depth, and other physical parameters are usually invoked to constrain the depths of models of conductivity. Figure 1 (inset) shows a model obtained by Porath²⁶ on the assumption that the conductive layer is identical with the seismic low-velocity layer. It shows one, non-unique model which fits the magnetovariation fields along the section SS at latitude 38° N. The section and map can be used together to gain an impression of conductive structure under the western region. Recent magnetotelluric soundings^{27,28} have verified that high conductivities occur at lower crustal depths, in general agreement with the section shown.

I have shown elsewhere²⁹ that the electrical conductivity distribution is highly correlated with heat flow and with the seismic low-velocity, high-attenuation layer. The conductivities required to model the magnetovariation fields are very high, and consistent with ionic conduction in a melt fraction in interconnected rock pores³⁰. While there are several other ways in which rocks can be highly conductive³¹, in the western US the close association of electrical conductivity with the seismic low-velocity layer, and with high heat flow, makes it very probable that the conductive layer is, in fact, a layer of the uppermost mantle containing a molten mineral fraction³². It is thus reasonable to regard Fig. 1 as a map and section of partial melting in the uppermost mantle. Shankland and others³³ have examined the properties required in a porous rock containing a melt fraction, to account for the electrical conductivities and seismic parameters observed.

Thus, there is substantial evidence for widespread partial melting in a layer, roughly 150 km thick, in the upper mantle under much of the US in a region bounded to the east by the east front of the Rocky Mountains and to the west by the Sierra Nevada. Furthermore, in this layer the rigidity modulus and estimates of *Q* are reduced, compared with the craton. As a first approximation, the crust and the lithosphere may be treated as

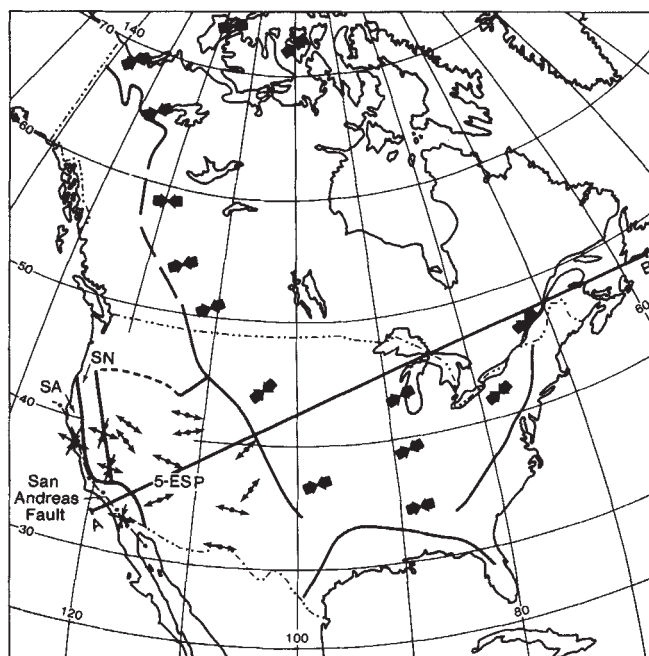


Fig. 2 Stress orientations in North America. Pairs of broad arrows show the directions of the greater horizontal principal stress in the craton. Two-headed arrows indicate the directions of the lesser horizontal principal stress in five extensional stress provinces (5-ESP). SN, Sierra Nevada stress province; SA, San Andreas stress province. Data sources: US and eastern Canada, ref. 37; western Canada, ref. 42; Arctic Islands, ref. 49. AB marks the great circle of the section in Fig. 3.

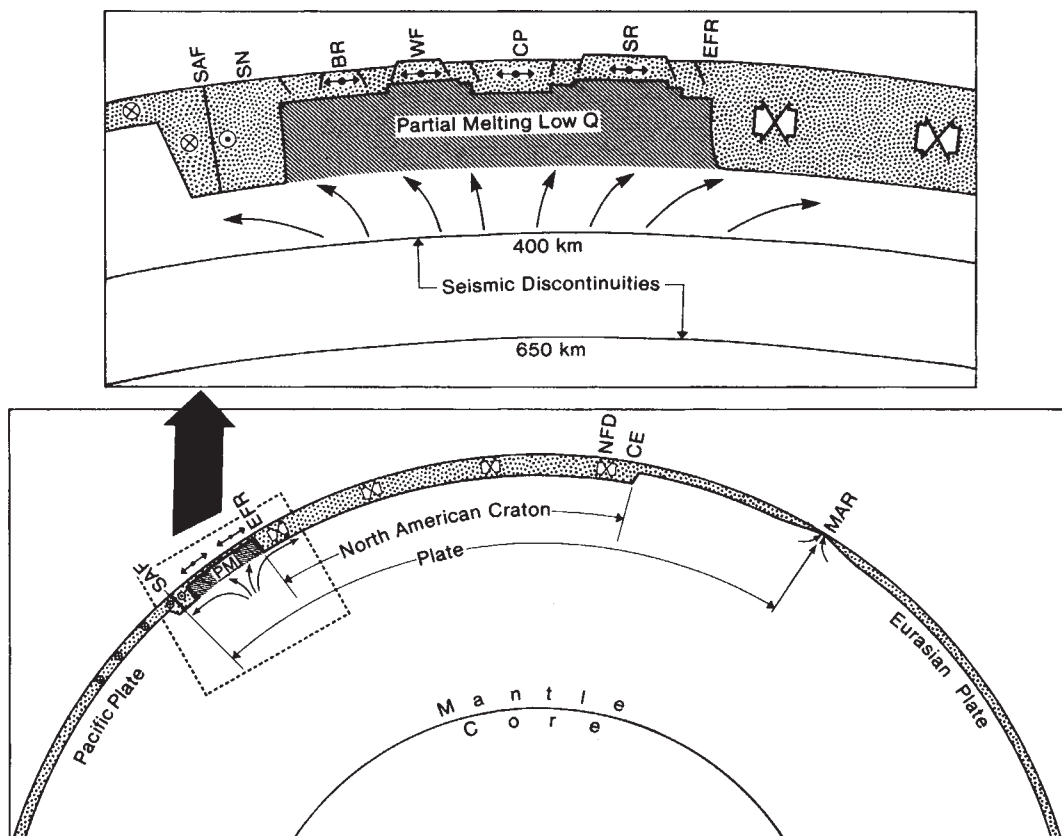
equivalent in the region of partial melting, and are represented as such in Fig. 3.

Mass transport of heat by an upflow of mantle material, formerly under the EPR and now under the continent, provides an explanation of all of the phenomena discussed above. However, heat was carried up in ascending melt from the former subduction, until about 20 Myr ago. There is now a crust ~30 km thick which can transmit heat only by conduction, and its thickness may have varied with time. It is therefore difficult to show that the existing layer of partially molten rock could not be a remnant from melt which came up from the plate before subduction ended. The change in character of the volcanics, from andesitic to basaltic composition, is difficult for proponents of the 'remnant' view but is not in itself conclusive. An entirely different line of evidence has become available which, I believe, resolves the ambiguity.

Stress orientations in North America

The orientation of the stress tensor in the Earth's crust, within a tectonic plate, contains important information on the forces driving that plate. In general, one principal stress is approximately vertical, that is, normal to the free surface; and fractures produce normal faults if σ_1 is vertical, strike-slip faults if σ_2 is vertical and thrust faults if σ_3 is vertical³⁴, where $\sigma_1 \geq \sigma_2 \geq \sigma_3$ are the principal stresses. For plate tectonics, special interest lies in the orientation and magnitudes of the two horizontal principal stresses, denoted S_H and S_h , with $S_H \geq S_h$. Crustal stress can be investigated by means of field studies of recent movements on faults and of igneous intrusions³⁵, and through overcoring strain relief techniques, hydraulic fracture experiments and earthquake source mechanisms³⁶. Using these methods in a study of crustal stress in the conterminous United States, Zoback and Zoback³⁷ have delineated 14 stress provinces. Within each province the directions and relative magnitudes of the horizontal principal stresses are nearly constant. The largest of these is their Mid-Continent Stress Province, which extends from the east front of the Rocky Mountains of Colorado and Wyoming to New England and the Atlantic provinces of Canada.

Fig. 3 A cartoon section, without radial exaggeration, along great circle AB in Fig. 2. The lithosphere is stippled. Stress symbols correspond to those in Fig. 2. SAF, San Andreas Fault; EFR, East Front of the Southern Rocky Mountains; NFD, Newfoundland; CE, continental edge; MAR, Mid-Atlantic Ridge. In the inset, SN, Sierra Nevada; BR, Basin and Range Province; WF, Wasatch Front; CP, Colorado Plateau; SR, Southern Rockies.



This province is characterized by a NE-SW direction of S_H , which is often greater than the vertical stress^{37,40}. Recently, this stress province has been shown to extend through the western Canadian sedimentary basin to the Arctic coast at the Mackenzie River delta, and is probably coextensive with the old craton of North America^{41,42}. The extension has been made through the recognition that a class of fractures in the walls of oil-wells, known as breakouts^{43,44}, are shear fractures which result from amplification by the oil-well of the stress difference in a stress field with $S_H \neq S_h$ (refs 45-47). According to this hypothesis^{45,46}, the fractures elongate the section of the borehole with its long axis aligned with the lesser horizontal stress S_h . It has been verified by comparing the stress orientations from the breakouts with other indicators of stress orientation in five stress provinces^{41,42,46-48}, and is widely accepted.

Directions of S_H in the craton are shown in Fig. 2 by pairs of arrows: each pair represents between 3 and 30 stress directions. Cox⁴⁹ has given breakout orientations from 15 oil wells in the Arctic Archipelago, of which 13 show the same NE-SW orientation of S_H as elsewhere in the craton. He has also determined the azimuths of breakouts in numerous wells in Quebec, which give S_H directions similar to the most easterly pair of arrows in Fig. 2.

The North American craton is under a stress field with remarkably uniform NE-SW orientations of S_H . As remarked elsewhere^{37,42}, this strongly suggests northeastward traction on the base of the plate. Such traction would arise if the plate were sliding to the south-west and experiencing viscous drag from a passive asthenosphere³⁷, or if northeastward flow in the underlying mantle were pushing the plate, again by viscous drag⁴². The latter case would apply if a divergent upcurrent were present in the mantle beneath the region west of the Rocky Mountains. The stress orientation in the craton cannot discriminate between these two possibilities.

The key to the problem is provided by the stress field in the region between the east front of the Rocky Mountains and the Sierra Nevada. Double-headed arrows in Fig. 2 represent directions of S_h , each from several stress orientations given by Zoback

and Zoback³⁷, in five of their stress provinces showing east-west extensional stress. These extensional stress provinces include the Basin and Range, Colorado Plateau and Rio Grande Rift. In all of them the dominant fault type is normal, which requires σ_1 to be vertical and $\sigma_3 = S_h$. It is not implied that east-west tensile stress exists except very near the surface; σ_3 is probably a small compression in most localities. A normal-fault type of stress field is what is required for uplift of the Colorado Plateau and Southern Rockies, and for development of the horst and graben topography of the Basins and Ranges.

This normal-faulting, extensional stress field is also what would be expected above an upcurrent in the mantle. It is, in fact, the stress field in the oceanic crust at ridges. Figure 3 shows an attempt to present this visually, for which purpose I have assumed lithosphere thicknesses of 250 km under the craton and 70 km below the sea floor. The thick cratonic lithosphere is controversial but the present argument does not depend on it. In the western region I suggest, in Fig. 3, that the seismic low-velocity, electrical high-conductivity uppermost mantle has reduced strength, and is essentially part of the asthenosphere. This is probably too simple but again is not essential to the argument.

The extensional stress field, between the Great Plains and the Sierra Nevada, cannot be understood by assuming that the North American plate is sliding over a passive asthenosphere to the south-west. On that model one would expect NE-SW compression to continue to the vicinity of the Sierra Nevada or even to the San Andreas fault system. Indeed if the lithosphere is thin above the western region (as shown in Fig. 3) one would expect the concentration of stress in it to produce increased NE-SW compression there. Instead, there is an extensional stress field. This requires vertical pressure from below which makes σ_1 vertical in the crust, as observed. Note that upward pressure exists now, as stresses propagate with the speeds of seismic waves. While the melt in the upper mantle could conceivably be a remnant from the subduction regime, say 25 Myr BP, it is difficult to believe that the uplift stress from such a melt remnant would not have been relieved in 25 Myr when postglacial

isostatic rebound times are shorter by a factor of 1,000.

The change from andesitic to basaltic volcanics ~25 Myr ago fits the hypothesis that a mantle upcurrent was overridden by the continent then. The stress pattern indicates that there is now upward pressure. Recent evidence⁵⁰ from surface wave velocities indicates low velocities of vertically polarized shear waves at depth 250 km under the presently spreading EPR and the San Andreas and Queen Charlotte Islands transform fault regions, with resolution insufficient to discriminate between the San Andreas fault and the Basin and Range Province. These three observations strongly support continuing mantle upflow under the continent. Other evidence, noted earlier, strongly suggests such upflow but can be explained otherwise.

Implications for plate dynamics

The prime question for plate dynamics is what form convection takes. Even if the plates slide over a passive asthenosphere, propelled by gravity sliding⁵¹ from the ridges ('ridge push') and, where there is a subducted leading edge which has not reached 650 km depth, by the excess weight of the subducted plate⁵² ('trench pull') there is convection with some undefined return path. This simplest model cannot, however, account for the persistence of upflow under western North America.

Convection in the mantle is required to move the plates and various types are under examination. Simplifications in the distribution of the tectonic plates⁵³ and in geoid highs and lows⁵⁴⁻⁵⁶ suggest deep mantle circulation, and whole-mantle convection is a model still favoured by some⁵⁷. However several lines of geochemical and geophysical evidence increasingly indicate a stratified mantle with change of chemical composition at the seismic discontinuity near depth 650 km (refs 58-62). Whereas convection may be expected both below and above the 650-km level, the two convective patterns may be independent or weakly coupled⁵⁶. Clearly, the upper mantle circulation will be of greater relevance to plate dynamics. Many models of convection in a 650-km layer describe square cells of comparable width which could not drive a plate thousands of kilometres wide. However, Houseman has shown⁶³ that convection cells of large width-to-depth ratio are possible if they are driven by a horizontal temperature difference such as would be expected between the mantle beneath an ocean ridge and a subduction. Anderson⁵⁸ has suggested alternatives to the various simplifying assumptions often adopted on convection. He suggests that melting may be more effective in producing the density change that drives convection rather than thermal expansion.

Evidence has been presented to show that an upward flow in the upper mantle, formerly under the EPR, is now under the Basin and Range and Colorado Plateau region of western North America. The mantle upflow may extend to western Canada, and the strip of low S_V velocity in Anderson's tomographic maps⁵⁰ suggests that it does. This would be consistent with the uniformity of stress orientation in the craton into the Arctic islands (Fig. 2). If the mantle upflow is present as I have argued, there are two points of interest related to convective models.

First, the upflow continued while the subducted plate (now the root of the Sierra Nevada) passed over it. It must, therefore, come from a depth near the 650 km discontinuity, as a minimum.

Second, the convective circulation has survived the effects of the continent overriding its upward limb for ~25 Myr. If one applies Houseman's type of convection, the operative temperature difference is that between colder mantle below the subductions on the north-west side of the Pacific, and hotter mantle, formerly below the EPR, now below western North America. Note that the upflow is not under the craton, whose great thickness of poorly-conducting lithosphere would probably change the flow rapidly⁵⁸. With the thin crust of the Basin and Range Province as conductive cover, the upflow may have boundary conditions not too different from those of the Rise sea floor.

It is not implied that all ocean ridges lie above convective upcurrents in the mantle. Spreading rates of ridges vary, and *a priori* it seems possible that fast-spreading ridges such as the

EPR lie above divergent mantle flow which drives them; whereas slowly-spreading ridges like the Mid-Atlantic Ridge may not be driven and may approximate to the pull-apart model. Gravity sliding will operate in both types, and even under a passive ridge partial melt will ascend, because its density is less than the mean value for the upper mantle, to form new lithosphere. In this case, however, the flow beneath the ridge will be convergent and only the sliding lithosphere itself exhibits divergent motion.

Conclusions

Observations of topography, seismology, heat flow, electromagnetic geophysics, petrochemistry and the orientation of the stress tensor in the crust, combine to support the view that an upcurrent exists in the mantle below western North America. This, in turn, implies that during the Tertiary, the EPR was a ridge of the active type, driven by a divergent upcurrent in the underlying mantle. Other ridges are probably of the passive type, but this has not been demonstrated.

For plate dynamics, a principal implication is that at least some tectonic plates are actively driven by mantle circulation. An interesting possibility is that the Pacific Plate is being driven by mantle convection towards the subductions which form its northwestern boundary. In such a system, if substantiated, the upward limb of the convection would lie under the EPR of southern and tropical latitudes, and under western North America.

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Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene

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Transcription of the c-fos proto-oncogene is greatly increased within minutes of administering purified growth factors to quiescent 3T3 cells. This stimulation is the most rapid transcriptional response to peptide growth factors yet described, and implies a role for c-fos in cell-cycle control. Transformation by c-fos may result from a temporal deregulation of this control.

ABERRANT expression of a variety of normal cellular genes (proto-oncogenes) can transform a normal cell into a tumour cell¹⁻³. Several of these genes encode protein products which are homologous to either a growth factor or a growth factor receptor. For example, the *sis* oncogene of the simian sarcoma virus displays striking homology with platelet-derived growth factor (PDGF)^{4,5}, a small polypeptide that stimulates the growth of connective tissue cells². A portion of the sequence of the epidermal growth factor receptor is virtually identical to another oncogene (*erb-B*) carried by avian erythroblastosis virus⁶. Moreover, several growth factor receptors, including those for PDGF⁷⁻⁹, epidermal growth factor (EGF)^{10,11} and insulin^{12,13}, as well as 10 different oncogene protein products³, share functional homology in that they are all associated with tyrosine kinase activity.

Much of the information about growth factors and their receptors has been obtained by stimulating BALB/c-3T3 fibroblast cells whose growth has been arrested by serum starvation to undergo a synchronous progression through the cell cycle by the addition of serum¹⁴. In the absence of serum, 3T3 cells enter a state (G₀) in which cell division and growth stop and many metabolic processes are reduced¹. The addition of serum initiates a complex series of events (G₁ phase), which culminates 14-20 h later in DNA synthesis (S phase) and cell division¹⁴. PDGF is the serum component that renders 3T3 cells competent to undergo DNA synthesis¹⁵⁻¹⁸, although additional components such as EGF and the somatomedins (progression factors) seem to be necessary to maintain cell viability¹⁹⁻²³. PDGF interacts with the external surface of cells by binding to a specific receptor protein in the plasma membrane²⁴ which activates a receptor-associated tyrosine kinase activity^{7-9,25}. Almost nothing is known, however, about the mechanisms by which these membrane changes lead to nuclear events such as the activation of specific cell-cycle genes and ultimately DNA replication.

The availability of cloned DNA probes for a variety of cellular genes has made it possible to examine the transcriptional events resulting from growth factor stimulation. Several virally encoded transforming proteins have normal cellular homologues that function in cell-cycle regulation⁴⁻⁶, suggesting that in general, proto-oncogenes might be involved in cell-cycle control. Such a possibility is supported by recent experiments^{26,27} demonstrating a cell-cycle dependence of the expression of the proto-oncogene *c-myc* mRNA. This gene encodes a nuclear protein²⁸ whose abnormal expression has been implicated in a wide variety of naturally occurring tumours²⁹⁻³⁷.

The *c-onc* genes, particularly those encoding nuclear antigens, might be differentially regulated at the transcriptional level during cell-cycle progression. We therefore examined the extent

of transcription of 17 different *c-onc* genes as a function of their position in the cell cycle, using a nuclear run-off transcription assay^{38,39}. We report here that stimulation of BALB/c-3T3 cells with defined growth factors induces a rapid transient increase in the transcription of the proto-oncogene *c-fos*, the normal cellular homologue of the transforming gene of FBJ-osteosarcoma virus⁴⁰⁻⁴². *c-fos* is a nuclear protein⁴³ of unknown function which is expressed at high levels in extra-embryonal tissues^{44,45}.

Analysis of *c-onc* transcription

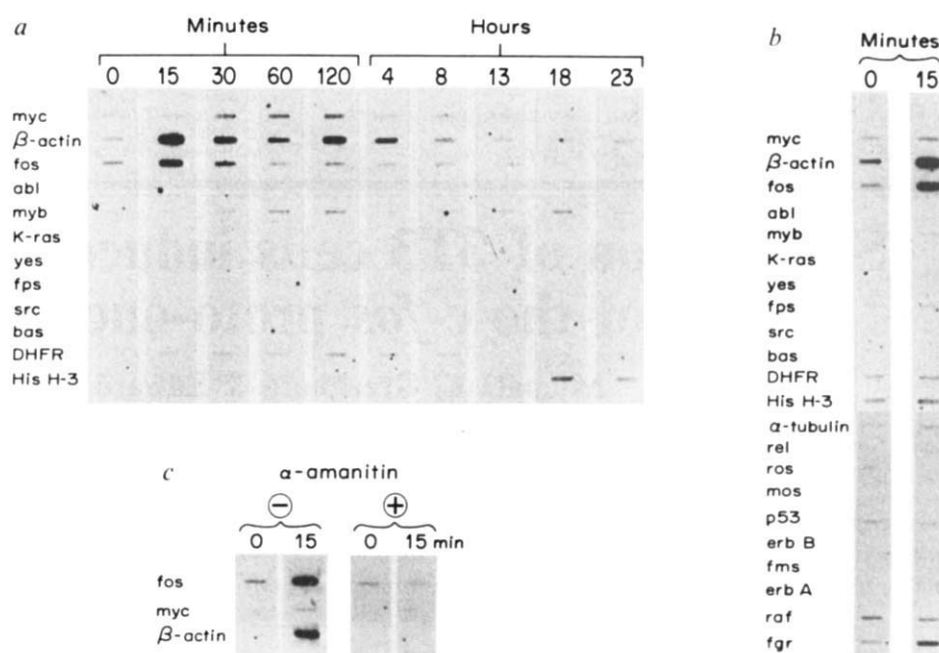
We measured the effect of a mitogenic signal on the transcription of a range of different *c-onc* genes using isolated nuclei from BALB/c-3T3 cells (Fig. 1). The nuclear run-off transcription assay was used to measure the level of transcription of specific genes in a variety of cell types^{39,40,46-50}. In this assay, new RNA transcripts are not initiated, but transcripts which are already initiated are faithfully elongated, giving a reasonably accurate measure of the level of transcription at the time of cell lysis^{38,39,51}.

Nuclei isolated from BALB/c-3T3 cells between 0 and 24 h after serum stimulation show a striking increase in the transcription of two genes, *c-fos* and actin, within 15 min of the initial stimulation of quiescent BALB/c-3T3 cells (Figs 1a, 2). *c-fos* transcription transiently increases more than 15-fold very soon after stimulation, returning to its initial levels within 30 min. No further changes in *c-fos* transcription are observed as the 3T3 cells continue to progress through the cell cycle from G₁ to S phase. The alteration in actin transcription appears more complex. The initial increase and subsequent decrease in actin transcription parallels that of *c-fos*. After about 60 min, however, actin transcription begins to increase again (Fig. 1a). This increase may correspond to the actin mRNA increase seen within 4 h of serum addition to growth-arrested 3T3 cells^{27,52}. These initial experiments use an actin cDNA plasmid that does not distinguish which of the several different cross-hybridizing actin genes^{53,54} display the cell-cycle dependent regulation of expression.

We also observe a small but reproducible increase in *c-myc* transcription after serum stimulation (Figs 1a, 2), which peaks approximately 2 h after stimulation and decreases significantly within 4 h. This experiment suggests that the previously reported growth-factor induced changes in *c-myc* mRNA^{26,27} are at least in part controlled at the level of transcription. In addition, the transcription of *c-myb* (encoding another nuclear antigen⁵⁵) appears to increase in parallel with *c-myc* (Fig. 1a). This result is inconclusive, however, because the *c-myb* plasmid DNA that was used, pVM2, also contains flanking cellular and viral sequences.

The increases in the transcription of *c-fos* and actin precede

Fig. 1 Transcriptional analysis of *c-onc* genes after stimulation of BALB/c-3T3 cells. *c-onc* gene plasmids bound to nitrocellulose were hybridized with 32 P-labelled run-off transcripts from nuclei isolated at different times after serum addition to confluent, growth-arrested BALB/c-3T3 cells. **a**, 23-h time course experiment analysing the transcription of 12 different genes. Increased transcription of *c-fos* and β -actin occurs within 15 min of serum addition, increased *c-myc* transcription occurs after 2 h and increased histone H3 transcription after 18 h. The changes in transcription are not due to hybridization of labelled RNA transcripts to non-specific DNA such as pBR322 sequences. No significant hybridization of 32 P-labelled RNA was detected for *onc* plasmid DNAs such as *v-abl* which contains a complete sequence of pBR322. Furthermore, when *c-fos* and actin sequences were cleaved from the pBR322 vector with restriction enzymes, Southern blotted and probed with 32 P-labelled RNA transcripts, significant hybridization to *c-fos*- or actin-specific fragments was detected but no hybridization to pBR322 sequences occurred (data not shown). The possibility that the actin and *c-fos* transcription signal is the result of cross-hybridization between these two genes was ruled out by a competition experiment in which preincubation of 32 P-labelled run-off transcripts with the actin plasmid, did not deplete the level of RNA transcripts hybridizing to *pv-fos-1* (data not shown). **b**, Analysis of the transcription of 22 different genes in growth-arrested 3T3 cells (0) and cells stimulated with calf serum for 15 min (15). **c**, Transcription of *c-fos*, *c-myc* and β -actin was measured in isolated nuclei in the presence (+) or absence (-) of $1 \mu\text{g ml}^{-1}$ α -amanitin. Nuclei were isolated from growth-arrested 3T3 cells (0) or 3T3 cells stimulated with calf serum for 15 min (15).



Methods: Cell culture: BALB/c-3T3 A31 cells obtained from the American Type Culture Collection were passaged to a density of 9×10^5 cells per 175-cm² tissue culture flask in 25 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Gibco), and allowed to grow to confluence over a period of 7–8 days at 37 °C, 10% CO₂. After 7 days in culture, the confluent monolayers had depleted the medium of serum growth factors and the 3T3 cells had growth arrested and entered a G₀ state as described previously¹⁴. The cells were then stimulated to progress synchronously through the cell cycle by removing the depleted medium and replacing it with fresh DMEM containing 15% calf serum. Early passage BALB/c-3T3 cells were used for all experiments. Nuclear run-off transcription assay: Medium was removed from one 175 cm² tissue culture flask of BALB/c-3T3 cells, the cells were washed three times with ice-cold phosphate-buffered saline (PBS), scraped in PBS and pelleted at 500g for 5 min. The cell pellet (1×10^7 cells) was resuspended in 1 ml NP40 lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% (v/v) NP40), incubated for 5 min on ice and centrifuged at 500g for 5 min. The supernatant was removed and used for the isolation of polyadenylated RNA. The nuclear pellet was washed once with 2 ml NP40 lysis buffer and centrifuged at 500g. The supernatant was discarded, the nuclei were resuspended in 100 μ l 50 mM Tris-HCl pH 8.3, 40% (v/v) glycerol, 5 mM MgCl₂, 0.1 mM EDTA and frozen in liquid N₂. The nuclei were thawed for the run-off transcription assay and mixed with 100 μ l reaction buffer (10 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 300 mM KCl, 0.5 mM each of ATP, CTP, and GTP and 100 μ Ci of [α - 32 P] UTP (760 Ci mmol⁻¹, NEN) and reacted at 30 °C for 30 min. The 32 P-labelled RNA was isolated as described elsewhere³⁹ with different conditions for hybridization to filters. Before ethanol precipitation, 32 P-labelled RNA was treated with a final concentration of 0.2 M NaOH for 10 min on ice. The solution was neutralized by the addition of HEPES (acid free) to a final concentration of 0.24 M and the RNA was precipitated with ethanol. After centrifugation, the 32 P-labelled RNA pellet was resuspended in 10 mM N-tris(hydroxymethyl)methyl 2-aminoethanesulphonic acid (TES), pH 7.4, 0.2% SDS, 10 mM EDTA at 5×10^6 c.p.m. ml⁻¹. This RNA solution was mixed with an equal volume of 10 mM TES pH 7.4, 0.2% SDS, 10 mM EDTA, 600 mM NaCl and 2 ml of the RNA solution was hybridized at 65 °C for 36 h to DNA immobilized on nitrocellulose. In a given experiment, each filter was hybridized with the same number of c.p.m. 32 P-labelled RNA. After the hybridization the filters were washed with several changes, $2 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M NaCl}$, 0.0125 M Na citrate pH 7) for 2 h at 65 °C and incubated at 37 °C in $2 \times \text{SSC}$ with 10 $\mu\text{g ml}^{-1}$ RNase A for 30 min. The filters were then washed again in $2 \times \text{SSC}$ at 37 °C for 1 h, air dried and exposed to Kodak SB-5 X-ray film. For binding to nitrocellulose, plasmids were linearized by restriction enzyme digestion and the DNA was denatured by incubation with 0.2 M NaOH for 30 min at room temperature, followed by neutralization with 10 volumes of $6 \times \text{SSC}$. The DNA was spotted onto nitrocellulose using the Schleicher and Schuell slot blot apparatus. 10 μg DNA was applied per slot. The plasmids used were provided by Dr Inder Verma (pVfos-1, v-fos⁶¹), Dr Larry Kedes (human β -actin-1, β -actin⁵⁴), Dr Kenneth Marcu (pM-c-myc-54, c-myc⁶²), Dr David Baltimore (pAB3sub3, v-abl⁷²), Dr J. Michael Bishop (pVM 2, v-myb⁷²; perb-A, v-erb-A⁷⁴, perb-B probe F, v-erb-B⁷⁴, pSRA, v-src⁷⁵), Dr Edward Scolnick (Merck, Sharpe and Dohme) (pHiHi-3, v-Ki-ras⁷⁶), Dr Hidesaburo Hanafusa (pFSV, v-fps⁷⁷), Dr George Vande Woude (pMS1, c-mos⁷⁸), Dr Howard Temin (peco-rel, v-rel⁷⁹), Dr Keith Robbins (pv-fgr1000, v-fgr⁸⁰), Dr Lui-Hai Wang (pv-ros, v-ros⁸¹), Dr U. Rapp (pv-raf E-H, v-raf⁸²), Dr Charles Scherr (pSM3, v-fms⁸³), Dr Steven Tronick (pHB1, v-bas⁸⁴), Dr M. Yoshida (pY73, v-yes⁸⁵), Dr Nicholas Cowan (pmca-1, α -tubulin, unpublished), Dr Arnold Levine (pp53-208, p53⁸⁶), Dr Robert Schimke (pDHFR11, dihydrofolate reductase⁸⁷) and Dr Nathaniel Heintz (pHh5B, hisH-3⁸⁸).

that of *c-myc* (Fig. 1) and occur during the time interval in which the 3T3 cells are becoming growth-factor independent^{19,22,23}. All these changes occur as the 3T3 cells progress from G₀ to G₁ and before the initiation of cellular DNA synthesis at ~14 h after serum stimulation, measured by the incorporation of ³H-thymidine into trichloroacetic acid (TCA) precipitable counts (Fig. 2). In similar conditions many other *c-onc* genes including *c-Ki-ras* whose viral counterpart encodes a GTP binding protein^{56,57} and *c-abl* which encodes a

tyrosine kinase⁵⁸, displayed barely detectable levels of transcription at all times after serum stimulation (Fig. 1a). Other sequences such as those for α -tubulin were transcribed at a low but constant level throughout the cell cycle and the transcription of the histone H3 gene was observed to increase at the time of DNA synthesis as reported previously (Fig. 1a)^{47–49}.

When 22 different genes were tested (Fig. 1b) only 3, *c-fos*, actin and an additional gene, *c-fgr* (ref. 59), exhibited a change in transcription 15 min after serum stimulation. *c-fgr* is the

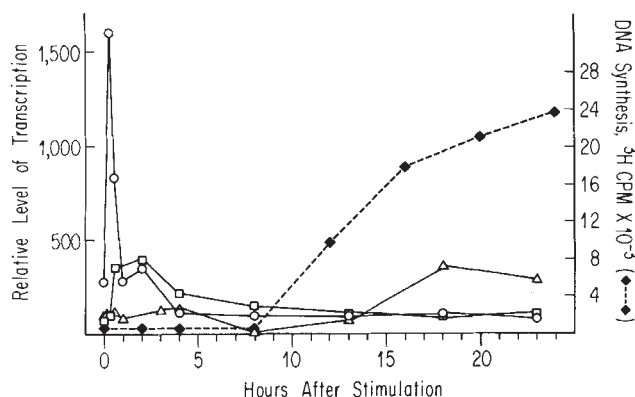


Fig. 2 Quantitation of transcriptional activation after stimulation of BALB/c-3T3 cells. The relative amounts of ³²P-labelled nuclear run-off transcripts hybridized to *c-fos* (○), *c-myc* (□) and histone H3 (△) plasmids bound to nitrocellulose, were determined by quantitative densitometric scanning of autoradiographs from Fig. 1a⁸⁹ (arbitrary units). DNA synthesis was measured after stimulating growth-arrested BALB/c-3T3 cells to divide by the addition of 15% calf serum as described in Fig. 1 legend. The incorporation of [³H]thymidine into DNA (■) was measured by pulse labelling cells with 90 μCi [³H]thymidine (2.0 Ci mmol⁻¹) for 2 h at the indicated times after serum stimulation. The medium was removed, cells washed three times with ice-cold PBS, and lysed with 0.2 M NaOH. An aliquot of the cell lysate was precipitated with TCA, collected on Whatman GF/A glass fibre filters and counted in toluene/omni-fluor scintillation fluid. In all the experiments reported in Figs 1–6, serum stimulation of quiescent BALB/c-3T3 cells resulted in a 20–80-fold increase in the incorporation of ³H-thymidine into TCA precipitable counts.

normal cellular homologue of *v-fgr*, the transforming gene of Gardner–Rasheed feline sarcoma virus. The *v-fgr* DNA we used as a probe is a hybrid gene containing a portion of actin as well as a tyrosine kinase sequence⁶⁰. Therefore, the increased hybridization in the *v-fgr* slot (Fig. 1b) probably reflects increased actin transcription. *c-fgr* tyrosine kinase gene transcripts may, however, also change.

Several control experiments were performed to establish the fidelity of the run-off transcription assay. The increased transcription of *c-fos* and actin within 15 min of serum addition is not simply due to an increase in overall transcription efficiency, because the transcription of a variety of other genes is unaffected by serum stimulation (Fig. 1b). Also, no reproducible differences in the total number of counts incorporated into RNA in isolated nuclei are observed during progression of 3T3 cells through the cell cycle (data not shown). Genes such as histones and actin display a cell-cycle dependent change in transcription consistent with previous results^{27,47–49,52}, further indicating that the isolated nuclei retain their transcriptional fidelity. The increased *c-fos*, *c-myc* and actin transcription seems to be the result of increased RNA polymerase II utilization, as α -amanitin completely blocked the transcription of these genes (Fig. 1c).

The plasmids used to measure *c-fos* and *c-myc* were oncogene specific. The *pv-fos-1* probe contains sequences entirely from the *v-fos* coding region and sequence homology to the corresponding *c-fos* sequences is >99%⁴². *pv-fos-1* recognizes a unique *EcoRI* restriction fragment in mouse DNA⁶¹, making it unlikely that our transcription assay has detected repetitive sequences due to cross-hybridization. Furthermore, the *pv-fos-1* plasmid hybridizes to a discrete 2.2 kilobase (kb) mRNA which is the size of *c-fos* mRNA⁴⁴. Moreover, *c-fos* lacks homology with any known cellular sequence⁴², making it unlikely that the observed increase in the transcription of both *c-fos* and actin reflects a homology between these two genes as appears to be the case for actin and *v-fgr*.

It was more difficult to establish the specificity of the *c-myc* transcriptional signal. The *pM-c-myc-54* plasmid used to

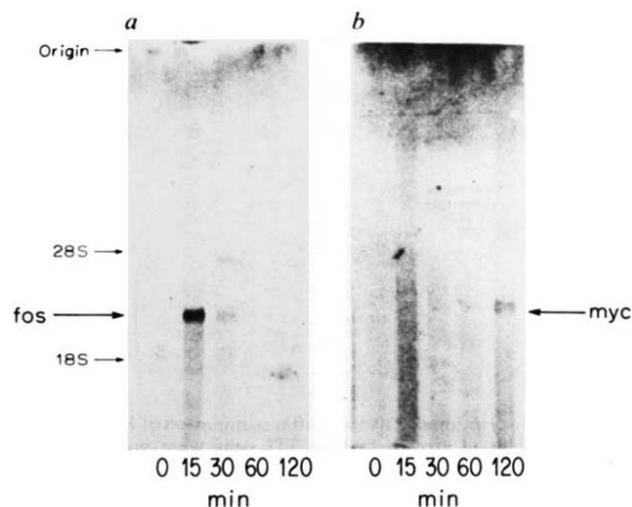


Fig. 3 Kinetics of *c-fos* and *c-myc* mRNA induction after serum stimulation of BALB/c-3T3 cells. Polyadenylated cytoplasmic RNA from 3T3 cells, isolated at various times after serum stimulation, was fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose, hybridized with ³²P-labelled *pv-fos-1* (a) or *pM-c-myc-54* (b) plasmids, and after washing exposed to SB-5 X-ray film. **Methods:** Polyadenylated RNA was isolated from the post-nuclear supernatant (see Fig. 1 methods). The supernatant (4 ml, from 4 × 10⁷ cells) was mixed with 0.2 ml 0.2 M vanadyl ribonucleoside complex (BRL) and centrifuged at 10,000g for 10 min at 4 °C. The supernatant was then mixed with 4 ml 0.2 M Tris-HCl pH 7.5, 0.44 M NaCl, 2% SDS, 25 mM EDTA containing 0.8 mg proteinase K (Worthington) and incubated at 37 °C for 30 min. After phenol and chloroform extractions, RNA was precipitated with ethanol. Pelleted RNA was resuspended in 10 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.2% SDS, denatured by heating to 65 °C for 5 min and RNA (~50 μg) applied to a 0.5-ml poly(U) Sepharose column as described previously⁹⁰. The polyadenylated RNA was fractionated on a 1% formaldehyde agarose gel and transferred to nitrocellulose following the procedure of Cheng-Kiang *et al.*⁹¹. The RNA bound to nitrocellulose was prehybridized overnight with buffer containing 1% glycine, 50% formamide, 5 × SSC, 50 mM NaH₂PO₄ pH 6.5, 250 μg ml⁻¹ sonicated salmon sperm DNA, 1 × Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin). The hybridization was carried out for 12–16 h in 10 ml 50% formamide, 2 × SSC, 40 mM NaH₂PO₄ pH 6.5, 200 μg ml⁻¹ salmon sperm DNA, 0.8 × Denhardt's, 10% dextran sulphate, 10 mM EDTA, 0.1% SDS, with 1 × 10⁸ c.p.m. ³²P-nick-translated plasmid (4 × 10⁸ c.p.m. per μg DNA)⁹².

measure *c-myc* transcriptional levels is a mouse cDNA, composed of the three *c-myc* exons inserted into the *PstI* site of pBR322⁶². This plasmid recognizes a *c-myc*-specific fragment and two *c-myc*-homologous sequences⁶². Although it is possible that the increased transcription observed using the *c-myc* probe is a result of increased transcription from one of these homologous *c-myc*-like sequences, this appears unlikely as the *pM-c-myc-54* plasmid detects a discrete *c-myc* mRNA of 2.4 kb which displays the expected cell-cycle dependent change in expression.

Stimulation of *c-fos*

To determine whether the increased transcription of specific genes after serum addition is followed by an increase in cytoplasmic mRNA, we isolated cytoplasmic RNA from quiescent and stimulated cells and analysed polyadenylated species (Fig. 3a). A prominent band of 2.2 kb appears 15 min after stimulation with a molecular weight equivalent to *c-fos* mRNA⁴⁴. The 2.2 kb mRNA level is highest 15 min after serum addition and decreases below detection within 60 min; the half life is thus very short.

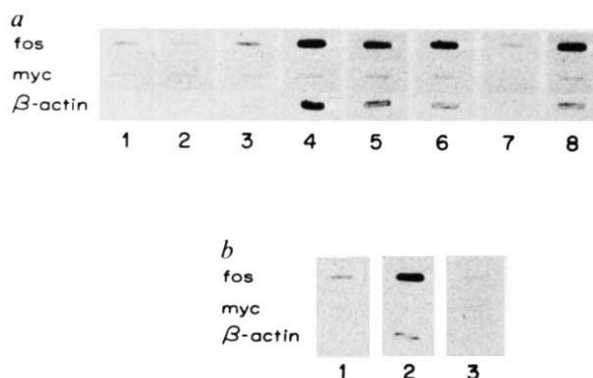


Fig. 4 Transcriptional activation after stimulation of BALB/c-3T3 cells with defined growth factors. 3T3 cells were grown to confluence and growth arrested as described in Fig. 1 legend. **a**, The medium was removed and cells were incubated for 15 min with: lane 1, the same depleted medium; lane 2, DMEM without calf serum; lane 3, DMEM containing 0.5% calf serum; lane 4, DMEM containing 15% calf serum; lane 5, DMEM containing 2.5 U ml⁻¹ partially purified porcine PDGF (Speywood); lane 6, DMEM containing 0.1 μ g ml⁻¹ fibroblast growth factor (Collaborative Research); lane 7, DMEM containing 10 μ g ml⁻¹ insulin (Sigma); lane 8, DMEM containing 0.26 μ g ml⁻¹ TPA (Sigma). **b**, The medium was removed and cells were incubated for 15 min with: lane 1, the same depleted medium; lane 2, DMEM containing 1.0 unit ml⁻¹ homogeneous porcine PDGF; lane 3, DMEM containing 0.1 μ g ml⁻¹ EGF (Collaborative Research). After stimulation with growth factor, cells were collected, nuclei isolated and run-off transcription assays performed. The ³²P-labelled RNA transcripts were hybridized with pv-*fos*-1, pM-*c-myc*-54 and pH β -actin plasmids immobilized on nitrocellulose as described in Fig. 1 legend. The radioactivity was visualized by autoradiography. 1 U of PDGF is the amount of growth factor needed per ml of DMEM to yield 50% maximal incorporation of ³H-thymidine into DNA in confluent growth-arrested Swiss 3T3 cells⁹³.

The kinetics of *c-fos* mRNA induction are quite different from *c-myc*. Consistent with previous results^{26,27}, virtually no *c-myc* mRNA is detectable in the cytoplasm until 2 h after serum addition, at which point a substantial increase in the 2.4 kb *c-myc* mRNA (Fig. 3b) is observed.

We next tested the ability of specific purified growth factors to induce the transcription of the *c-fos* gene. When growth-arrested 3T3 cells are incubated in serum-free medium, no change in the level of *c-fos* transcription is observed (Fig. 4a, lane 2). If the serum-free medium is supplemented with various factors at concentrations that effectively generate the competence phase of the mitogenic response (Fig. 4)^{19-22,63}, a reproducible increase in *c-fos* transcription is observed within 15 min. Hormones that do not significantly stimulate competence in BALB/c-3T3 cells, but do maintain cell viability²¹, cause no significant change in *c-fos* transcription (Fig. 4). In contrast with *c-fos*, the level of transcription of most other genes is not detectable after 15 min of treatment, although *c-myc* transcription is induced by PDGF and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) within 1 h of growth factor addition (Fig. 5). Actin transcription is also stimulated with these purified factors in parallel with *c-fos* although the level of stimulation appears to be less than that observed using whole serum (Fig. 4). The kinetics of *c-fos* stimulation are identical when cells are treated with PDGF (Fig. 5a), TPA (Fig. 5b) or serum (Fig. 1a). The increased levels of *c-fos* nuclear transcripts are apparent as early as 5 min after serum addition; the *c-fos* transcription level peaks after 10 min, and begins to decrease by 15 min (Fig. 6).

Conclusions

The stimulation of *c-fos* transcription within 5 min of treatment of quiescent 3T3 cells with PDGF is the earliest transcriptional

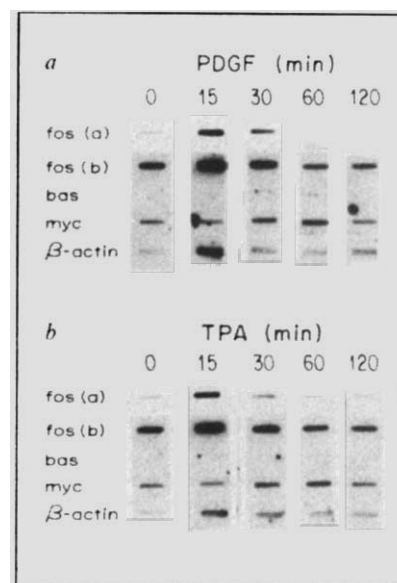


Fig. 5 Kinetics of *c-fos* transcriptional activation after stimulation of BALB/c-3T3 cells with PDGF and TPA. Nuclei were isolated from serum starved cells and 3T3 cells stimulated for 15, 30, 60 and 120 min with homogeneous porcine PDGF. **a**, 1 U ml⁻¹ DMEM; **b**, TPA 0.26 μ g ml⁻¹ DMEM. ³²P-labelled RNA transcripts were prepared as described in Fig. 1 legend and were hybridized with pv-*fos*-1, pbas, pM-*c-myc*-54 and pH β -actin DNA immobilized on nitrocellulose. The radioactivity was visualized by autoradiography. *c-fos*(a) filters were exposed for 18 h; *c-fos*(b), *c-bas*, *c-myc* and β -actin filters exposed for 5 days.

event known to occur in these cells in response to a growth factor; in other cells, factors such as glucocorticoid hormones have been shown previously to stimulate the transcription of murine mammary tumour virus genes with equally rapid kinetics⁶⁴.

The increased transcription of *c-fos* and *c-myc* genes is quickly followed by a substantial increase in the respective mRNAs. The transcriptional changes in *c-fos* and *c-myc* do not appear as large as the concomitant alterations in mRNA, perhaps because of differences in the sensitivity of the two assays, or because of changes in *c-fos* and *c-myc* mRNA stability. We have not established whether the cell-cycle dependent changes in expression result in comparable changes in the levels of the respective protein products. In the case of *c-fos*, this question is particularly significant because expression of the *c-fos* protein may be regulated by sequences at the 3' end of the mRNA which, in some conditions, appear to inhibit translation of the RNA into protein⁶⁵.

The rapid activation and subsequent repression of *c-fos* transcription coupled with the short half life of the *c-fos* mRNA could explain why the mRNA is expressed during a very brief interval, ~30 min, of the G₀/G₁ transition and also why *c-fos* mRNA has been barely detectable in many nonsynchronized cell types⁴⁴. If all cycling cells were to exhibit a transient increase in *c-fos* each time they pass through G₁, for a total of 15 min (~1/100th of the cell cycle), then in an asynchronous population of cells the levels of *c-fos* would be as much as 100-fold lower than the levels detected 15 min after synchronously stimulating cells to divide.

Our finding that the transcription of a proto-oncogene is rapidly stimulated in response to a growth signal raises questions about the role of this gene in the normal cell cycle, oncogenesis and transcriptional induction; for example, how a growth factor such as PDGF acts externally on the plasma membrane to induce gene transcription within 5 min. PDGF binding to its receptor activates a tyrosine kinase, which suggests that a protein modification such as phosphorylation could be involved in the transmission of the growth signal to the nucleus. This idea is

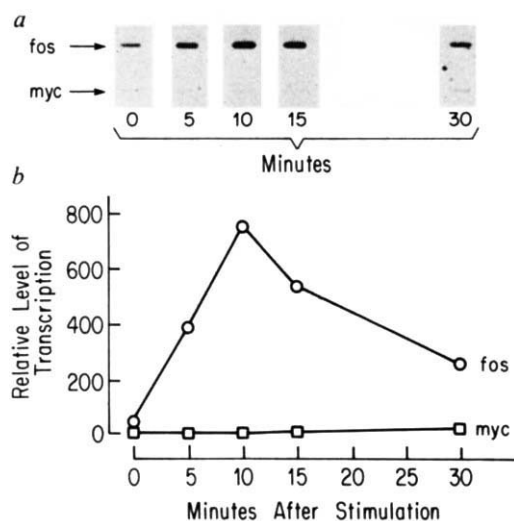


Fig. 6 Kinetics of *c-fos* transcriptional activation after serum stimulation of BALB/c-3T3 cells. Nuclei were isolated from serum starved 3T3 cells and from 3T3 cells stimulated with 15% calf serum for 5, 10, 15 and 30 min. 32 P-labelled RNA run-off transcripts were prepared as described in Fig. 1 legend and were hybridized with pv-fos-1, pM-c-myc-54 and pH β -actin DNA immobilized on nitrocellulose. *a*, Radioactivity visualized by autoradiography; *b*, relative signal intensities calculated by densitometric scanning of the autoradiographs.

supported by the observation that TPA, which activates both protein kinase C^{66,67} and tyrosine kinase⁶⁸⁻⁷⁰, also activates *c-fos* and *c-myc* transcription.

Although the time course of activation of the *c-fos* and actin genes can be clearly distinguished from *c-myc*, it is not known whether *c-fos* or actin activation is a prerequisite for activating *c-myc* or other genes. PDGF induces *c-myc* mRNA levels even in the presence of protein synthesis inhibitors²⁶, suggesting that the prior stimulation of *c-fos* may not be required to induce *c-myc* transcription. Our kinetic experiments suggest that different factors may be involved in the regulation of *c-fos* and *c-myc* and that these genes may be members of two distinct classes of inducible genes. By differential screening of a cDNA library prepared from cells stimulated with PDGF for 4 h, a set of induced genes have been identified⁷¹ into which *c-myc* may fall²⁵. *c-fos* may be a member of a new class of early response genes, with actin and other unidentified genes; they could have a crucial role in committing a cell to enter G₁. Unlike *c-fos*, some of the genes might not produce a dominant transforming phenotype and therefore might not be detected by current assays for oncogenes.

We speculate that an early response gene such as *c-fos*, whose deregulation can result in cell transformation and uncontrolled proliferation, might be a gene that directly controls normal cell growth, for example by activating the cells' progression from G₀ into G₁. Nevertheless, a direct effect of *c-fos* expression on cell-cycle progression remains to be shown. The activation of *c-fos* may be necessary for commitment to DNA replication and cell division, but it appears not to be sufficient. *c-fos* transcription is activated by competence factors in the absence of progression factors (Fig. 4); these conditions will generate an initial mitogenic signal but are not sufficient to induce cellular DNA synthesis¹⁹⁻²².

A variety of different mechanisms have been proposed to explain how normal cellular oncogenes might be altered so that they cause transformation, such as the overproduction of a normal gene or the mutation of a *c-onc* gene³. One proposal is that expression of high levels of *c-fos* protein in an inappropriate cell type might result in transformation⁶⁵. Our finding that *c-fos* exhibits cell-cycle dependent changes in transcription supports

a different possibility, that transformation by *c-fos* results at least in part from a deregulation of its temporal control. The expression of *c-fos* throughout the cell cycle rather than during a specific stage of the G₀/G₁ transition may be one mechanism of transformation. The continuous expression of *c-fos* might prevent a cell from ever entering a quiescent state, thus yielding a population of continually dividing cells.

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Differentiation of F9 teratocarcinoma stem cells after transfer of *c-fos* proto-oncogenes

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Transfer of mouse or human c-fos proto-oncogenes into F9 teratocarcinoma stem cells results in expression of c-fos mRNA and protein. This is accompanied by the appearance of morphologically altered cells which express several specific markers characteristic of differentiated cells, suggesting that c-fos plays a role in cellular differentiation.

RECENT analyses of the structure of retroviral oncogenes and of the expression of their cellular homologues (*c-onc* genes or proto-oncogenes) strongly support the hypothesis that the products encoded by such genes are directly linked with cellular growth control^{1–6}. Amino acid sequence homologies suggest that the *c-sis* gene probably encodes the platelet-derived growth factor^{1,2} and the *c-erb-B* gene product appears to be identical to the epidermal growth factor receptor³. Expression analyses have shown that the *c-myc* gene product may be associated with the initiation of normal cellular proliferation: high levels of *c-myc* transcripts were detected specifically during the early G₁ phase of the cell cycle when either quiescent lymphocytes⁴ or fibroblasts⁵ were stimulated to proliferate. In agreement with these conclusions, *c-myc* expression was seen to be down-regulated when teratocarcinoma stem cells (F9)⁵ or immature haematopoietic cells (HL60)⁶ were induced to differentiate into non-proliferative cell types. Terminal differentiation of HL60 cells was also accompanied by a decrease of *c-myc* expression⁶.

Another category of *c-onc* genes appears to be represented by *c-fos* whose gene product may be associated with functions in differentiated cells^{7–10}. Transcription of *c-fos* at high levels was detected during prenatal development in specific tissues, notably amnion and visceral yolk sac and mid-gestation fetal liver^{7–10}. The level of *c-fos* expression in the fetal membranes increased markedly at late stages of gestation⁸. In postnatal tissues, expression of *c-fos* at high levels was observed only in haematopoietic cells¹⁰. In cells of the monomyelocytic lineage high levels of *c-fos* mRNA were detected specifically at late stages of differentiation¹⁰.

We have started to introduce *c-fos* genes into a variety of different cell types and into fertilized mouse eggs in order to analyse a potential function of the *c-fos* gene product in differentiation processes. We describe here the results of a study using 'nullipotent' F9 teratocarcinoma stem cells¹¹ as recipients for the introduction of *c-fos* genes. Teratocarcinoma stem cells have been used as a model system to study differentiation and development¹², and the introduction of selectable and nonselectable genes into various established embryonal carcinoma cell lines has been accomplished¹³. F9 stem cells do not differentiate

spontaneously in culture¹¹, but treatment with retinoic acid and dibutyryl cyclic AMP can lead to endoderm-like differentiation^{14,15}. Depending on culture conditions, parietal or visceral endoderm-like cells are formed in the presence of the chemical inducers, as judged by the expression of several specific differentiation markers. It has also been shown that nerve growth factor can induce adrenergic neuronal differentiation in F9 cells treated with retinoic acid and dibutyryl cyclic AMP¹⁶. We show here that the expression of either the human or mouse *c-fos* gene following DNA-mediated gene transfer into F9 stem cells results in a drastic alteration of cell morphology and proliferative potential associated with the onset of expression of several differentiation markers.

Morphological alteration of F9 cells

In F9 stem cells, the level of *c-fos* expression is normally very low¹⁷. To analyse whether an increased expression of the *c-fos* gene product affects the differentiation state of these cells, either

Table 1 Quantitative evaluation of transfection experiments

DNA	Ratio <i>c-fos</i> : pSV2- neo (μg)	Total no. of colonies†	Fraction of morpho- logically altered colonies (%)
<i>c-fos</i> (human) + pSV2-neo	10:10	300	15
<i>c-fos</i> (human) + pSV2-neo	15:5	85	30
<i>c-fos</i> (human) + pSV2-neo	20:1	80	70
<i>c-fos</i> (mouse) + pSV2-neo	20:1	100	50
<i>c-fos</i> (human) digested with <i>NcoI</i> * + pSV2-neo	5:5	75	0
<i>c-fos</i> alone	10	0	0
pSV2-neo alone	5	75	0

* Cleaves within *c-fos* coding region.

† Values are from at least two independent experiments and are expressed per 10⁶ transfected cells.

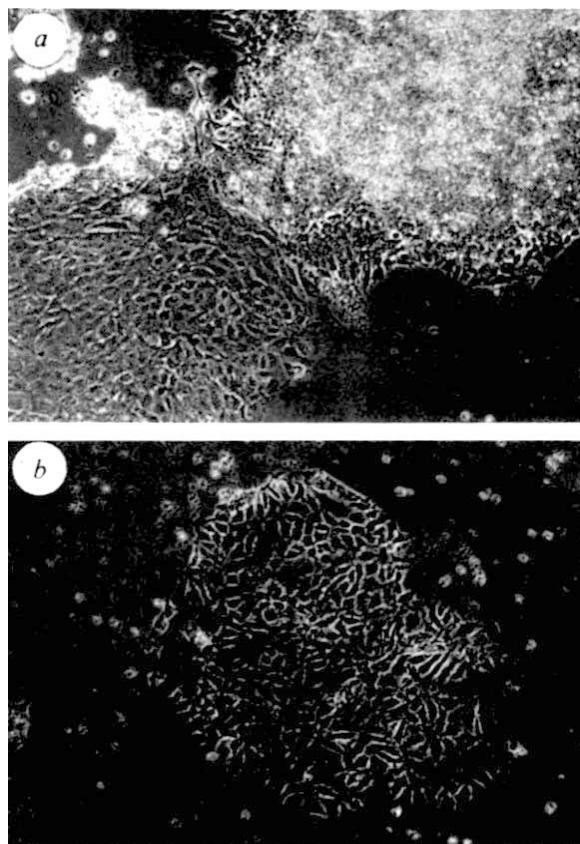


Fig. 1 Phase-contrast microscopy of F9 cells 10 days after co-transfection of *c-fos* and pSV2-neo and selection in G418-containing medium. *a*, The upper right colony is a typical F9 stem cell clone whereas in the lower left side, part of a characteristic *c-fos*-altered F9 clone is visible. *b*, A *c-fos*-altered F9 cell clone with the typical flat, epithelial-like morphology.

Method: DNA transfections were performed essentially as described^{31,33,34}. The CaPO₄-precipitated DNA (20 µg human *c-fos* plasmid and 1 µg pSV2-neo) was added to 10⁶ F9 cells and selection with G418 (1 mg ml⁻¹) was started 48 h later. Photographs were taken at magnification ×100.

the human¹⁸ or the mouse¹⁹ *c-fos* gene and a plasmid (pSV2-neo)²⁰ containing a bacterial neomycin resistance gene were co-transfected into F9 stem cells. After selection in geneticin (G-418) containing medium for about 8–10 days, two types of colonies were detected with both *c-fos* genes: clones of cells showing the normal F9 stem cell morphology and colonies of greatly enlarged, flat cells growing in an epitheloid fashion (Fig. 1*a, b*). The maximum clone size of these morphologically altered cells was about 400 cells, although colonies consisting of less than 10 cells were also observed. No proliferative activity (as judged by clonal expansion) could be detected after 10–12 days in selective medium, whereas geneticin-resistant F9 stem cell clones continued to grow. The fraction of morphologically altered colonies was clearly dependent on the ratio of *c-fos*/pSV2-neo DNA used for transfection (Table 1). No clone of morphologically altered cells was obtained when the neomycin plasmid was transfected alone, or when the *c-fos* plasmid was cleaved within the coding region of *c-fos* prior to co-transfection (Table 1). No biological effect on F9 stem cells has been observed on transfer and expression of *v-myc* and *v-src*, which were linked to the metallothionein promoter region (M. Karin, personal communication). These results strongly suggested that the introduction of intact *c-fos* genes was responsible for the observed morphological alteration of F9 stem cells.

To obtain further evidence in support of this conclusion, DNA from transfected cultures was analysed for the presence of

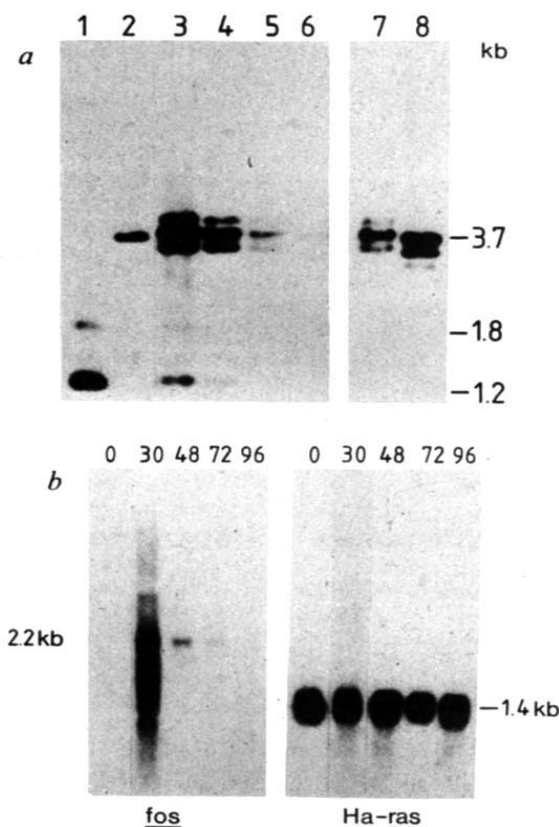


Fig. 2 *a*, Presence of exogenous *c-fos* DNA sequences in *c-fos*-altered F9 cells, 12 days after co-transfection of the human *c-fos* gene and pSV2-neo and subsequent selection in geneticin-containing medium. DNAs were digested with *Pvu*II and analysed by Southern blotting²¹ using a *v-fos*-specific probe. Lane 1: normal F9 cells, 10 µg DNA; lane 2: normal human fibroblasts, 10 µg DNA; lanes 3–6: *c-fos*-altered F9 cells (experiment I, transfection of 10 µg *c-fos* and 10 µg pSV2-neo), 3 µg, 1 µg, 0.3 µg and 0.1 µg DNA (from left to right); lane 7, same as lane 3, but shorter exposure to facilitate comparison with lane 8 which contains *c-fos*-altered F9 cells (experiment II, transfection of 20 µg *c-fos* and 1 µg pSV2-neo). *b*, *c-fos* transcripts in transfected F9 stem cells. Poly(A)⁺ RNA was isolated³⁵ 0, 30, 48, 72 and 96 h after transfecting 20 µg of the mouse *c-fos* plasmid¹⁸ onto 10⁶ F9 cells, separated on formaldehyde-containing agarose gels (8 µg per lane) and analysed by Northern blotting for transcripts hybridizing to a *v-fos* or *v-Ha-ras*-specific probe, respectively, as described^{8,35,37}. The *Ha-ras*-specific probe was used to show that the pattern of expression observed with the *fos* probe was not due to quantitative or qualitative differences in the RNA samples analysed. After longer exposure of the same blot hybridized to the *v-fos*-specific probe, 2.2-kb transcripts were clearly visible at 96 h.

exogenous *c-fos* sequences (about 14 days after co-transfection of the normal human *c-fos* gene and the pSV2-neo plasmid). On digestion with *Pvu*II, the *v-fos*-specific probe used in this study recognized a fragment of 3.7 kilobases (kb) in human DNA and two fragments of 1.8 and 1.2 kb in mouse DNA (Fig. 2*a*). When mass cultures containing approximately 50% morphologically altered colonies were analysed by the Southern blot procedure²¹, an average value of at least 20–50 copies per cell was determined for the exogenous human *c-fos* genes (Fig. 2*a*). When DNA from transfected, but morphologically normal, F9 cell clones was analysed by the same technique, only few intact copies of exogenous *c-fos* sequences were detected (R.M. and E.W., unpublished observation). These results suggest that *c-fos* expression in the morphologically altered F9 cells may result from low-level transcription of many exogenous *c-fos* genes (see below).

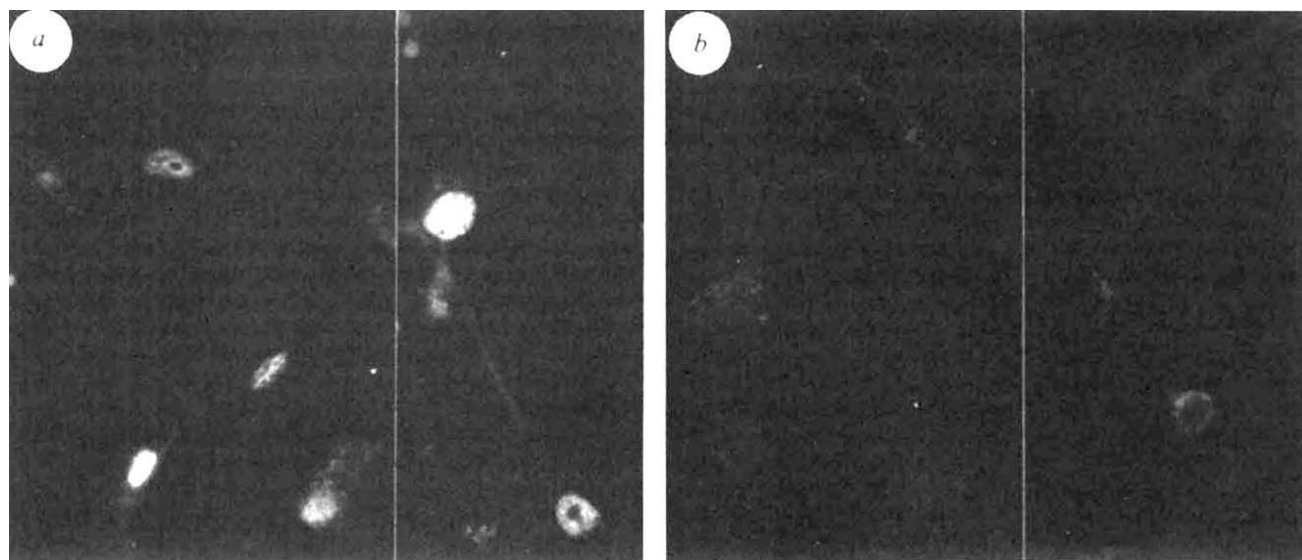


Fig. 3 Expression of *c-fos*-related nuclear protein in *c-fos*-altered F9 cells, 12 days after co-transfection of the human *c-fos* gene and pSV2-neo and subsequent selection in geneticin-containing medium. Double-antibody immunofluorescence using *fos*-specific TBRs^{22,23} and a rhodamine isothiocyanate-conjugated sheep anti-rat-IgG antibody (provided by J. Adamkiewicz, University of Essen, FRG) was carried out on *p*-formaldehyde-fixed and Triton-permeabilized cells as described by Curran *et al.*²³. *a*, *fos*-specific TBRs showing a clear nuclear staining²³; *b*, preimmune serum showing only non-specific cytoplasmic fluorescence. Similarly, no specific nuclear staining was observed with TBR serum on normal F9 stem cells (not shown). Two different areas of the same culture are shown in both *a* and *b*.

Increased expression of *c-fos* mRNA

To examine whether the *c-fos*-induced morphological alteration was associated with elevated expression of *c-fos* mRNA, we analysed total poly(A)⁺-containing RNA from cultures at different times after transfection of the mouse *c-fos* gene (thus including in the analysis all cells which had not taken up DNA). High levels of *c-fos*-related transcripts relative to untransfected F9 cells could be detected as early as 30 h after transfection (Fig. 2*b*). In addition to the normal *c-fos* mRNA of 2.2 kb, which has previously been described to be present in various mouse and human tissues⁷⁻¹⁰, transcripts of heterogenous size producing a smear of *c-fos*-containing RNA were observed (Fig. 2*b*). At later times the level of *c-fos*-related RNA decreased and the *c-fos* transcripts predominantly occurred as normal 2.2-kb mRNA (Fig. 2*b*). These changes in the level and size of *c-fos* transcripts are apparently not due to variations in the RNA preparations, as neither quantitative nor qualitative changes could be observed when the same RNA blot was hybridized to a *v-Ha-ras*-specific probe (Fig. 2*b*). Our observations on *c-fos* expression might therefore reflect the elimination of extrachromosomal *c-fos* sequences or of unstable *c-fos* integration sites which could produce aberrant transcripts. The decrease of *c-fos* expression at later times could also occur because normal F9 stem cells may have a growth advantage over those cells which have taken up and express exogenous *c-fos* genes. Our results, however, demonstrate that *c-fos* sequences, on transfection into

F9 stem cells, are transcribed at significant levels, leading to an over-expression of *c-fos* mRNA that is similar in size to the normal transcript.

Expression of *c-fos* protein

Exogenous *c-fos* genes transferred by transfection into F9 cells are transcriptionally active (Fig. 2*b*), but *Nco*I cleavage in the *fos* coding region abolishes this activity of the human *c-fos* gene (Table 1). These observations suggest that the altered F9 cell morphology may be induced by the *c-fos* protein. Using a tumour-bearing rat serum (TBRs), it has been reported recently that both *v-fos* and *c-fos* gene products are expressed as nuclear phosphoproteins in virus-transformed fibroblasts and in primary cultures of late-gestation mouse amnion cells, respectively^{22,23}. This *fos*-specific antiserum was used here to investigate whether *c-fos* protein was expressed in the morphologically altered F9 cells. Because of the relatively low number of *c-fos*-altered F9 cells available, this analysis was only possible by using an *in situ* immunostaining procedure. As shown by immunofluorescence (Fig. 3), a strong nuclear staining was detected in most of the morphologically altered F9 cells. Only relatively weak nonspecific cytoplasmic staining (no nuclear fluorescence) was observed when either TBRs was used on normal F9 stem cells or when non-immune serum was applied to *c-fos*-altered F9 cells (Fig. 3). Therefore, our findings strongly suggest that *c-fos*

Table 2 Differentiation markers of *c-fos*-altered F9 cells compared with those of F9 stem cells, trophoblastic cells and endodermal cells

Cell type	Marker					Deposited collagen type IV ^{26,27}
	TROMA-1 ^{25,26}	TROMA-3 ^{25,26}	Laminin ^{25,26}	AFP ²⁶	Fibronectin ²⁵	
F9 stem cells	—	—	—	—	(+)	(—)
<i>c-fos</i> -transformed F9 cells	+	+	—	—	(+)	(+)*
Trophoblastic cells	+	+	—	—	?	?
Primitive endoderm	?	?	+	—	+	+
Parietal endoderm	+	(+)	+	—	—	+
Visceral endoderm	+	—	(+)	+	+	(+)

—, No expression detectable; (+), low level of expression; +, high level of expression (—, (+) and + correspond to —, +, more than one + in ref. 26).

* Variation among different clones from very low to high levels.

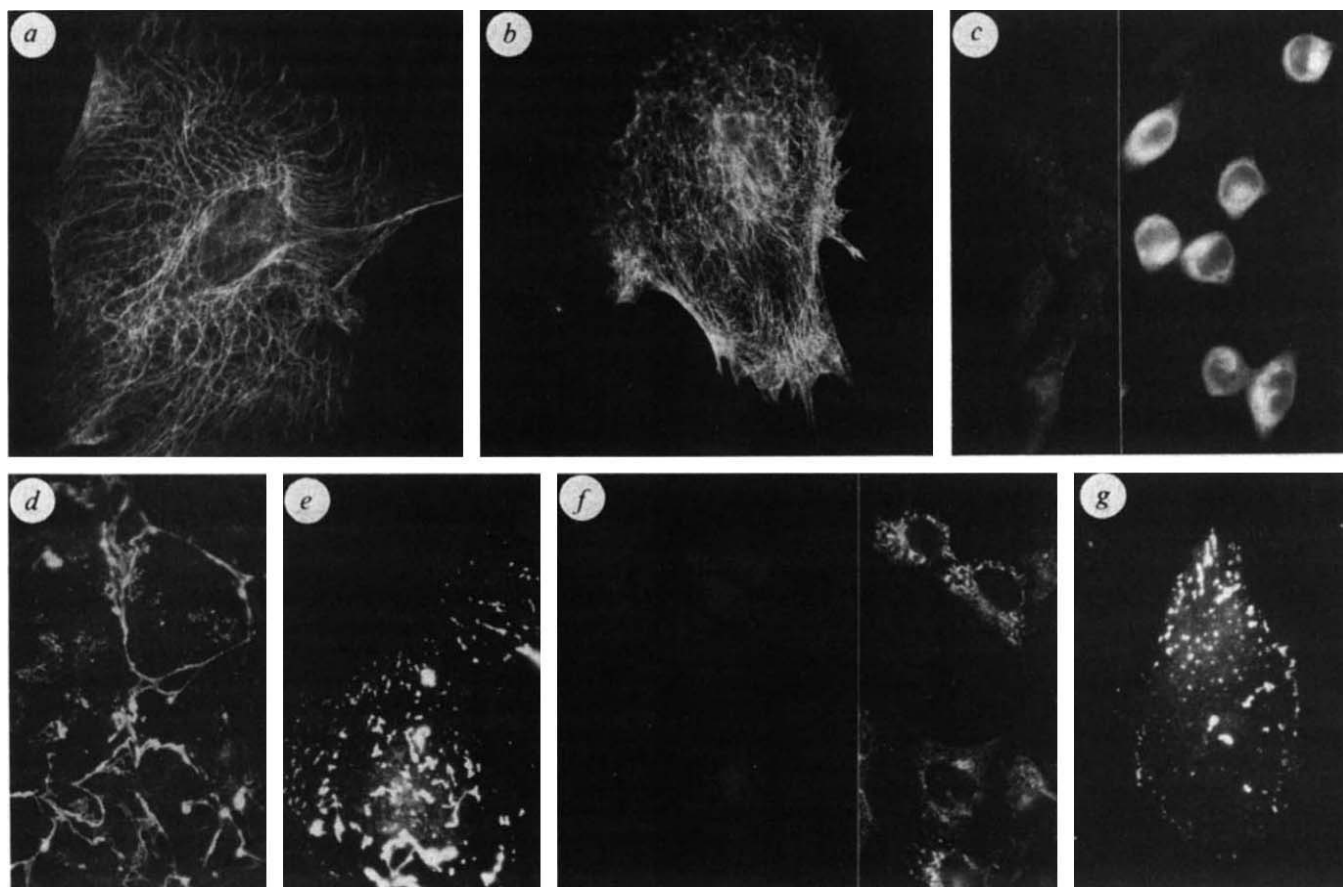


Fig. 4 Expression of differentiation markers in *c-fos*-altered F9 cells 12 days after co-transfection of the human *c-fos* gene and pSV2-neo and subsequent selection in geneticin-containing medium. Cells were fixed with Sante Marie reagent (ethanol/glacial acetic acid, 99:1 v/v) (*a–f*) or *p*-formaldehyde (*g*) and processed by double-antibody immunofluorescence as described elsewhere³⁸. *a, b*: Mouse monoclonal antibodies TROMA-1 (*a*) and TROMA-3 (*b*) recognizing intermediate filaments²⁵ (provided by R. Kemler, Tübingen, FRG) on *c-fos*-altered F9 cells. *c*, Rabbit antiserum against AFP in *c-fos*-altered F9 cells (left) and in rat hepatoma cells (DTH2) as a positive control (right) (cells obtained from D. Paul, Hamburg, FRG; antiserum provided by E. Adamson, La Jolla, California). *d, e*: Rabbit antiserum against collagen type IV (given by K. von der Mark, Martinsried, FRG) on *c-fos*-altered F9 cells. *d*, Shows a clone of cells; *e* shows two single cells. *f*, Mouse monoclonal antibody against laminin (obtained from R. Kemler, Tübingen, FRG) on *c-fos*-altered F9 cells (left) and on the parietal endoderm-like teratocarcinoma cell line PYS-2 (as a positive control, right). *g*, Rabbit antiserum against hamster fibronectin (provided by H. Beug, EMBL; originally obtained from R. Hynes, MIT, Boston) on a *c-fos*-altered F9 cell. The second antibody was as in Fig. 3 (in *a, b, f*) or rhodamine isothiocyanate-conjugated sheep anti-rabbit IgG (Dakopatts, Hamburg, FRG) (*c, d, e, g*). Microscopic magnification $\times 400$.

protein is synthesized in morphologically altered F9 cells. This observation is of particular relevance because other cell types may not efficiently express unmodified transfected *c-fos* genes²⁴. Although these data do not yet prove a causative role for *c-fos* protein in the morphological alteration of F9 stem cells, such a function seems possible because *c-fos* mRNA was detected as early as 30 h after transfection. Thus, it seems that *c-fos* expression precedes morphological alteration rather than vice versa.

Expression of differentiation markers

To investigate the nature of the morphologically altered F9 cells in further detail, we analysed the expression of a variety of differentiation markers in these cells. These studies were particularly designed to include those markers known to be switched on in F9 cells induced to differentiate by retinoic acid treatment^{14,15,25–27}. The most dramatic change we observed was the appearance of an ordered intermediate filament network in the morphologically altered F9 cells (Fig. 4*a, b*). This network, which is entirely absent from normal F9 stem cells (data not shown; see ref. 25), was recognized by two monoclonal antibodies reacting with different intermediate filament components: TROMA-1 (Fig. 4*a*) and TROMA-3 (Fig. 4*b*). These antibodies have both been reported to bind to intermediate filament proteins, which are first detectable in tropho-ectodermal cells

of the blastocyst²⁵. While TROMA-1 antibodies also react strongly with both visceral and parietal endodermal cells, the specificity of TROMA-3 is more pronounced; this antibody shows a positive reaction (though weaker than TROMA-1) only on parietal endoderm (Table 2)^{25,26}. In addition, both antibodies react with the primitive gut and fetal liver as well as with the mesoderm and endoderm-derived epithelia in adult animals. Although our results obtained with TROMA-1 and TROMA-3 already suggest the alteration by *c-fos* of F9 stem cells into an epithelioid cell type (in accord with the change in cell morphology; Fig. 1), the analysis of a larger number of differentiation markers was required to obtain a clearer picture of the cell type represented by the *c-fos*-altered F9 cells.

Figure 4*c, f* shows that the endodermal markers α -fetoprotein (AFP; expressed in visceral endoderm²⁶) and laminin (expressed predominantly in parietal endoderm²⁶) could not be detected in the morphologically altered F9 cells, although cell lines serving as positive controls showed strong reactions: the parietal endoderm-like cell line PYS-2²⁵ (Fig. 4*f*) and retinoic acid-induced F9 cells (data not shown) with monoclonal anti-laminin antibodies (Fig. 4*f*), and the rat hepatoma cell line DTH2 (D. Paul, personal communication) with anti-AFP serum (Fig. 4*c*).

Two other extracellular matrix proteins were found to be deposited in the morphologically altered F9 cells, fibronectin

(Fig. 4g) and collagen type IV (Fig. 4d, e)^{26,27}. Fibronectin was detected as extracellular patches of protein (Fig. 4g) and as deposits on the plastic surface of the culture dish (not shown) at levels similar to that found in F9 stem cells. Collagen type IV was found in an intracellular form (Fig. 4d), in extracellular fibres within cell clones (Fig. 4d) and in patches on single cells (Fig. 4e). The pattern and level of collagen type IV expression was subject to some variation when different clones were compared.

Conclusions

Mouse and human *c-fos* genes are expressed in a stage-, tissue- and cell type specific fashion which has led to the hypothesis that products encoded by these *c-onc* genes may have a role in cellular differentiation processes⁷⁻¹⁰. One approach for testing this hypothesis is to transfer transcriptionally active genes into various undifferentiated cell types, in particular stem cells and fertilized eggs, and to analyse the biological effect of the exogenous gene products. In the present study we have shown that the introduction via transfection of *c-fos* genes into F9 teratocarcinoma cells gives rise to colonies of cells which are morphologically distinct from F9 stem cells, and which possess phenotypic properties characteristic of differentiated cells. The *c-fos*-altered F9 cells share several markers with trophoblastic and endodermal cells (in particular the intermediate filament proteins TROMA-1 and TROMA-3 as well as collagen type IV; Table 2)^{25,26}. On the other hand, several markers diagnostic of retinoic acid-induced F9 cells and characteristic of either parietal endoderm (laminin)^{25,26} or visceral endoderm (α -fetoprotein)²⁶ are not expressed in the *c-fos*-transfected, differentiated F9 cells. Thus, it seems that the differentiation pathways promoted by retinoic acid and *c-fos* are distinct events. Whether the *c-fos*-transfected F9 cells represent a normal cell type that has not yet been identified or whether an aberrant type of differentiation is triggered by *c-fos* is unknown. It is also possible that *c-fos* induces the expression of a particular set of genes, and that the acquisition of a terminal stage of differentiation requires the expression of another set of genes not controlled by *c-fos*. Nevertheless, our findings provide the first evidence that transfer of a single cellular gene can promote a cellular differentiation process, either by inducing differentiation in uncommitted stem cells or by conferring on committed cells present in the mass population of F9 cells the ability to pass through a predetermined differentiation pathway, for example by providing a maintenance function specifically required by differentiated cells.

Another intriguing aspect of this study is that *c-fos* sequences

introduced into F9 stem cells were found to be expressed at both the mRNA and protein levels (Figs 2b, 3). Recently, it has been shown that *c-fos* genes transferred into fibroblastic cells are not efficiently expressed unless part of the 3'-untranslated region is removed from the transfected gene²⁴. Such control mechanisms apparently do not exist in amniotic cells and, as shown here, also seem to be absent from *c-fos*-induced differentiated F9 cells. F9 stem cells could thus represent an invaluable tool for investigating the mechanisms controlling *c-fos* mRNA and protein expression.

Our present observations, taken together with previous findings on *c-fos* expression in normal development, suggest a participation of the *c-fos* gene product in specific cellular differentiation processes. However, it should be emphasized that such a putative role of *c-fos* would not necessarily preclude its association with growth control mechanisms, a function suggested by the fact that the inappropriate expression of *c-fos* can lead to neoplastic transformation (uncontrolled cell proliferation)²⁴. It is noteworthy that both differentiation-inducing properties and growth-promoting activities have been observed for nerve growth factor¹⁶ and the *v-abl* gene product^{28,29}.

To elucidate the nature of the *c-fos*-altered F9 cells in greater detail, it is important to obtain larger numbers of these differentiated cells; this might be achieved by improving culture conditions (for example, to compensate for a possible requirement of the *c-fos*-altered cells for specific factors), by introducing 'immortalizing' genes^{30,31} into the differentiated cells (to overcome a possible block of cell proliferation associated with terminal differentiation) or by using *c-fos*- gene constructs, allowing regulation of expression via inducible promoters³² (in case the constitutive expression of high levels of *c-fos* expression is detrimental to the cell). To address these problems, we are currently introducing *c-fos* genes under the control of the metallothionein promoter³² into various other stem cells and fertilized mouse eggs, either alone or together with the gene coding for the polyoma virus large-T antigen³⁰. By using such inducible *c-fos* gene constructs, it may also be feasible to examine whether the promotion of differentiation by *c-fos* requires the selection of pre-existing committed cells in the mass population of F9 stem cells.

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Accretion disk limit cycle mechanism in twin-degenerate interacting binaries

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The twin-degenerate interacting binary stars are thought to consist of two pure helium white dwarfs in close orbit. The candidates are G61-29 (also GP Com), HZ 29 (also AM CVn) and, tentatively, PG1346+082¹. Large variations in the optical light of PG1346+082 suggest variable accretion onto the star gaining mass. Such behaviour in the light curves of the classical dwarf novae is thought to be caused by either episodic mass overflow from the mass-losing star^{2,3} or the instability in the accretion disk^{4,5}. Here we investigate the latter mechanism, and explore the non-thermal equilibrium limit cycle behaviour of the accretion disk expected for a twin-degenerate interacting binary system.

G61-29 is a system containing two white dwarfs orbiting each other with a period of 46.5 min. Nather *et al.*⁶ interpret this system as consisting of a low-mass white dwarf transferring matter to its more massive companion through the inner lagrangian point. They find that the hydrogen-to-helium ratio is at least a factor of 1,500 lower than its solar value. It is also generally accepted that HZ 29 consists of two white dwarfs in close proximity⁷⁻⁹. The rate of mass transfer is thought to be orders of magnitude higher than for G61-29 (refs 6, 8). If the mass-losing star is also a low-mass white dwarf in contact with its Roche lobe, the orbital period would be of the order of 1/2 h, given the size considerations for white dwarfs.

Smak¹⁰ has pointed out that the disk limit cycle mechanism¹¹⁻¹⁷ hypothesized to account for the recurrent bursts seen in dwarf novae should also be applicable to twin-degenerate interacting binaries. This instability mechanism results from changes in the vertical disk structure which occur when the midplane temperature is near the peak in the Rosseland opacity. For a density of $\rho = 10^{-7} \text{ g cm}^{-3}$ this peak occurs at $T \approx 15,000 \text{ K}$ for solar composition material¹⁸ and $T \approx 30,000 \text{ K}$ for pure helium material¹⁹. The effective temperature of the disk corresponding to the onset of instability is $T_{\text{eff}} \approx 6,500 \text{ K}$ for the former¹¹⁻¹⁷ and $T_{\text{eff}} \approx 9,000 \text{ K}$ for the latter¹⁰. A comparison of vertical structure computations^{10,20} shows that in pure helium disks the instability occurs at a surface density which is a factor of 7.5 larger than that in disks composed of solar matter because of the higher temperatures.

The strongest evidence for dwarf nova-like nature of these objects is the resemblance of their spectra to those seen in the classical dwarf novae. The eruption spectra of dwarf novae show strong absorption lines and their light curves exhibit flickering. In HZ 29 one sees^{8,9} He absorption lines and flickering. Also, emission features are seen in dwarf novae during the quiescent periods between the outbursts—just as one sees⁶ emission lines of He in G61-29.

Preliminary observations of PG1346+082 appear to be at least consistent with the notion that it too contains two helium white dwarfs orbiting at close range (D. E. Winget, and R. E. Nather, personal communications). The average spectrum of PG1346+082 is devoid of hydrogen and strongly resembles that of HZ 29. As in HZ 29 the absorption features are shallower than those seen in lone white dwarf spectra. Observations of this object are preliminary and no light curves have been published. However, variations of 2–3 mag seen in the optical light from this system seem to hint at episodic disk flow onto the accreting star. Were this true, one would be faced with the prospect that $\dot{M}_T$ does, in fact, lie in an 'unstable' range¹⁰. What would be the expected amplitudes, durations, and recurrence times for bursts produced in such a system?

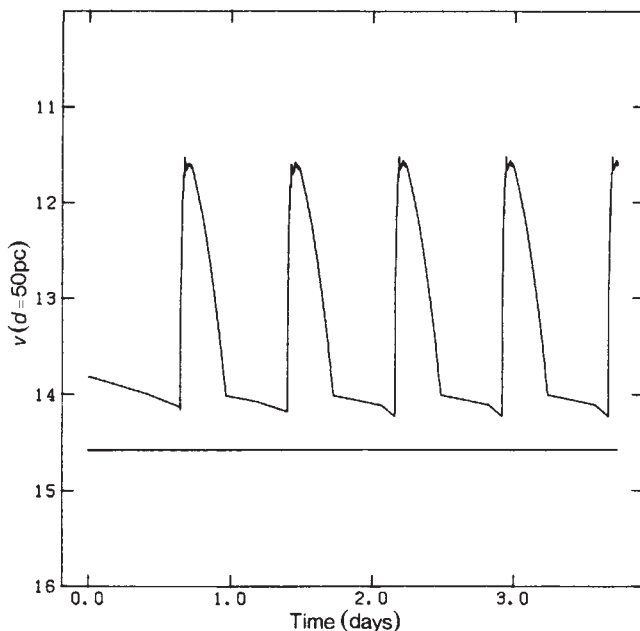


Fig. 1 A series of bursts for $\alpha_{\text{cold}} = 0.05$, $\alpha_{\text{hot}} = 0.2$, and $\dot{M}_T = 5 \times 10^{-10} M_{\odot} \text{ yr}^{-1}$. The horizontal line represents the light from a typical white dwarf. The vertical axis is in units of apparent visual magnitude. The system quickly relaxes to a periodic behaviour with widely separated bursts occurring.

In an attempt to answer these questions we have employed a generalized time-dependent, non-thermal equilibrium accretion disk model developed for studying the classical dwarf novae. The mathematical formulation is similar to that being used elsewhere^{20,21-24}. The exact method used²⁵ involves the α parameterization for the viscosity and consists of simultaneously solving for the temporal evolution of surface density and disk temperature.

Preliminary comparisons of theory with observation^{25,26} using dwarf novae systems seem to demand $\alpha_{\text{cold}} \approx 0.05$ and $\alpha_{\text{hot}} \approx 0.2$. We take the inner disk edge to be at $5 \times 10^8 \text{ cm}$, and the outer edge at $4 \times 10^9 \text{ cm}$. Mass is added to the disk at a constant rate at $2.5 \times 10^9 \text{ cm}$ —an appropriate injection radius for $P = 1/2 \text{ h}$. A radial grid of 40 points is used. At the inner edge we set $\Sigma = 0$, and at the outer edge we set $v_r = 0$ (where v_r is the radial flow speed). We assume a face-on disk in computing the light curves presented below.

We use the opacities of Heubner *et al.*¹⁹ which characterize matter with the composition $X = 0$, $Z = 0.001$ (where X is the hydrogen mass fraction and Z is the metal mass fraction). Power law scalings for the critical surface densities associated with the limit cycle are taken from computations using solar composition material¹⁶. These surface densities have to be multiplied by 7.5 for the helium-rich matter considered here.

We have computed a series of bursts for a wide range of values of the secondary mass transfer rate $\dot{M}_T$. Figures 1 and 2 show a series of bursts for representative low and high values of $\dot{M}_T$. The horizontal line represents the luminosity of a white dwarf with effective temperature, $T_{\text{eff}} = 30,000 \text{ K}$ and radius $r_{\text{wd}} = 5 \times 10^8 \text{ cm}$. The varying curve gives the disk contribution. We take the distance to be $d = 50 \text{ pc}$. In quiescence the optical depth through the disk vertically is $\tau \approx 1$ at all radii, and the disk temperature is $T \approx 10,000 \text{ K}$. In outburst, the disk effective temperature varies between about 20,000 K and 50,000 K. The optical flux from the disk rises about two magnitudes in going from quiescence to outburst.

Even for very low $\dot{M}_T$ we still find bursts occurring. For $\dot{M}_T = 2 \times 10^{-11} M_{\odot} \text{ yr}^{-1}$ (corresponding to G61-29) the bursts

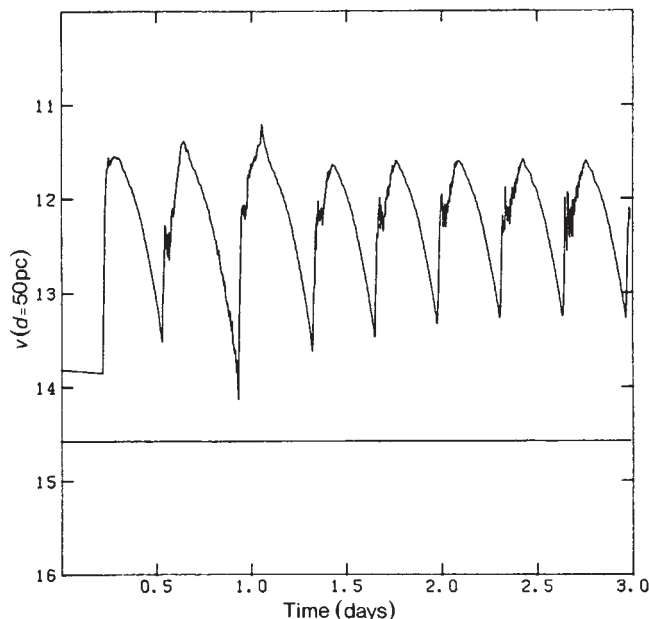


Fig. 2 A series of bursts for $\dot{M}_T = 1.5 \times 10^{-9} M_\odot \text{ yr}^{-1}$. Other parameters are as in Fig. 1. After three bursts the system settles to a repetitive pattern with closely spaced bursts.

happen once every 10 days and last about 5 h. Hence for the parameters chosen we predict a duty cycle of about 2%. So far this system has only been seen in the 'low' state. As $\dot{M}_T$ is increased the interval between bursts decreases but the duration of the burst itself increases slightly. The bursts shown in Fig. 1 for which $\dot{M}_T = 5 \times 10^{-10} M_\odot \text{ yr}^{-1}$ occur about once every 16 hours. There is still a sizeable quiescent period. Figure 2 depicts the system behaviour for $\dot{M}_T = 1.5 \times 10^{-9} M_\odot \text{ yr}^{-1}$. The bursts now occur so rapidly that the inner part of the disk is still in the high state when the next burst is initiated at larger radii. For even higher $\dot{M}_T$ the inner disk is always in the high state. Only the outer disk alternates between the low and high states. The disk light oscillates within a few tenths of a magnitude of $m_v = 12$. For $\dot{M}_T$ corresponding to HZ 29 the entire disk is in steady-state.

The burst amplitudes to be expected in a helium twin-degenerate interacting binary system are about three magnitudes, or less if $\dot{M}_T$ is large. The time between bursts is hard to predict from a theoretical standpoint without knowing $\dot{M}_T$. Indeed, a knowledge of the recurrence time should place limits on $\dot{M}_T$. If bursts are seen in one of these systems, the disk apparent magnitudes presented here should enable one to infer a distance, given an assumption for the disk inclination.

I thank Don Winget, Ed Nather, Rick Hessman, and Matt Wood for bringing PG1346+082 to my attention and for discussing the existing observations of this object. I also thank Don Winget for making available the Heubner *et al.*¹⁹ opacities, Doug Lin and Greg Shields for discussions involving the nonthermal equilibrium model, and especially Craig Wheeler for useful discussions of accretion disk physics and critical reading of this manuscript. This research was supported in part by NASA grant NSG 7232.

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Boltzmann's ultraviolet cutoff and Nekhoroshev's theorem on Arnold diffusion

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The greatest difficulty with classical statistical mechanics may be the fact that some degrees of freedom do not seem to attain the energy expected from the equipartition principle. This is typical of the vibrational modes in molecules and of the high frequencies in the electromagnetic radiation (problem of the ultraviolet cutoff). Boltzmann¹ had the intuition that an explanation might be provided if each degree of freedom were to have a characteristic relaxation time until equilibrium, and that such a time should greatly increase with frequency; he spoke in terms of relaxation times of days or centuries. This possibility was seriously considered by Rayleigh and Jeans², but they could not produce a clear classical mechanism to explain the freezing of the high frequency degrees of freedom; so, the equilibrium concept of the ultraviolet cutoff provided by quantum mechanics was accepted, and Boltzmann's hypothesis was forgotten. Here we point out that a general framework for understanding the ultraviolet cutoff in Boltzmann's dynamical sense in a classical context seems to be provided by Nekhoroshev's³ theorem on Arnold diffusion.

The Komogorov–Arnold–Moser (KAM) theorem^{4–9} on the existence of invariant tori in nearly integrable Hamiltonian systems was stated in 1954, the same year in which Fermi *et al.*¹⁰ found that a classical nonlinear string did not relax to equilibrium within the time then available to the computer. In the KAM theorem one concentrates on results which are valid for infinite times, and this imposes a strong limitation to the set of initial data, which are restricted to the complement of a dense open set. This is a serious obstacle to a physical interpretation. In Nekhoroshev's theorem, however, where the evolution of the system is considered only for finite (but large) times, one obtains estimates which are valid in open sets of phase space, and not only on the invariant tori.

Nekhoroshev considers an analytical Hamiltonian $H(I, \phi) = H^0(I) + \varepsilon H^1(I, \phi)$ for a system with s degrees of freedom, where $I = (I_1, \dots, I_s)$ and $\phi = (\phi_1, \dots, \phi_s)$ are action-angle variables, H^1 is 2π -periodic in $\phi_1, \dots, \phi_s$, $\varepsilon \ll 1$ is a perturbative parameter, and H^0 satisfies certain 'steepness conditions' (for example, H^0 is convex). Then his main theorem continues:

Let $0 < \varepsilon < \varepsilon^0$. Then for every solution $I(t), \phi(t)$ of the

system with Hamiltonian $H^0 + \varepsilon H^1$ one has

$$|I(t) - I(0)| < \mathcal{J} \varepsilon^b \quad (1)$$

for all $t \in (0, T)$, where

$$T = \tau \frac{1}{\varepsilon} \exp\left(\frac{1}{\varepsilon}\right)^a \quad (2)$$

with suitable positive constants a, b depending on $H^0, \mathcal{J}$ and τ being natural units, of action and time respectively, entering the problem.

Here $|\cdot|$ is the Euclidean norm in R^s . The actions are thus 'frozen' up to very long times, which increase exponentially with decreasing ε , if the perturbation is small enough. The dimensional constants $\mathcal{J}$ and τ are widely arbitrary, and, as often used in the mathematical literature, they were set equal to unity in Nekhoroshev's paper.

But what interests us here is his extrapolation to systems of coupled harmonic oscillators. In such cases, one considers a Hamiltonian of the form

$$H(p, q) = \sum_{j=1}^s \frac{1}{2} \omega_j (p_j^2 + q_j^2) + \sum_{k \geq 3} H^{(k)}(q)$$

where $p = (p_1, \dots, p_s) \in R^s$ and $q = (q_1, \dots, q_s) \in R^s$ are cartesian coordinates around an elliptic equilibrium point, the constants $\omega_1, \dots, \omega_s$ being the harmonic frequencies and $H^{(k)}$ a polynomial of order k in the variables q ; no small parameter ε is needed in the Hamiltonian, and the smallness is measured in terms of the Euclidean distance from the fixed point. As the quadratic part of the Hamiltonian does not satisfy the steepness conditions required in Nekhoroshev's theorem, one adapts the Hamiltonian by a preliminary change of variables which is the same used to apply KAM theorem⁸. Specifically, we assume that among the harmonic frequencies $\omega_1, \dots, \omega_s$ there are now low-order resonances, that is, a positive integer l exists such that no relations of the form $\sum_{j=1}^s k_j \omega_j = 0$ are satisfied, $k_1, \dots, k_s$ being integers with $0 < |k_1| + \dots + |k_s| \leq l$. Then, through a sequence of $l-2$ Birkhoff's transformations, and using the new variables I, ϕ , where $I_j = \frac{1}{2}(p_j^2 + q_j^2) + \dots$, the Hamiltonian takes the form $H'(I, \phi) = H^0(I) + H^1(I, \phi)$, where H^0 is in general, steep and H^1 starts with terms of order $|I|^{1/2}$. One has to take into account the fact that any Birkhoff's transformation produces a restriction of the domain around the fixed point where the new Hamiltonian is defined; thus, for any given set of harmonic frequencies, one could, in principle, find an optimal l . In any case, the new Hamiltonian H' can be defined for small enough actions, say for $|I| < A$, with a given 'critical action' A . Then one can apply the theorem quoted above, and still obtain the estimates in equations (1) and (2), the only difference being that ε has to be replaced by a quantity ε_{eff} depending on the initial data and defined by

$$\varepsilon_{\text{eff}} = \left(\frac{|I(0)|}{A} \right)^{1/2}$$

This is given in Nekhoroshev's paper with A set equal to unity.

Such a mathematical result seems strongly to indicate that the high frequency degrees of freedom are frozen. Indeed, let us consider a very special initial condition, such that only one oscillator, say the j th one, is excited, that is, one has $I_i(0) = 0$ for $i \neq j$. In terms of the harmonic energies E_j of the oscillators with harmonic frequencies ω_j , because of the relation $E_j = \omega_j I_j$ which holds in a first approximation, one has then

$$\varepsilon_{\text{eff}} \approx \left(\frac{|E_j(0)|}{A \omega_j} \right)^{1/2},$$

and the theorem guarantees that such oscillator is 'frozen', that is, it has small fluctuations of energy, up to a time T_j which, as seen from equation (2), is exponentially increasing with frequency. In this sense, the theorem seems to provide a general mechanism for freezing of the higher frequencies, as advocated by Boltzmann. In addition, this actually occurs for energies

below some critical energies proportional to frequency. The latter possibility was indeed conjectured more than 10 yr ago, on the basis of an analogy of critical energy with quantum zero-point energy, taking into account the adiabatic invariance of the action¹¹⁻¹².

There are several limitations to these considerations. First, the class of initial conditions to which Nekhoroshev's theorem directly applies is not broad enough, because one also ought to consider initial conditions with some oscillators below, and others above, the respective thresholds. Furthermore, in the KAM theory, and thus also in Nekhoroshev's theorem, there are extremely small lower estimates for the thresholds, even for situations with few degrees of freedom as, for example, in molecules. This difficulty is enhanced for most systems of interest in statistical mechanics, which involve many degrees of freedom, because such lower estimates tend rapidly towards zero as the number of degrees of freedom tends to infinity (in the thermodynamic limit). In this connection, we note that the available lower estimates in KAM theory are highly non-optimal; in addition, it seems that the estimates of the thermodynamic limit would be improved if they were restricted to special classes of physically-relevant perturbations, such as those corresponding to short-range interactions (low connectance, in the terminology of refs 13, 14); in particular, this should be the case for systems of particles coupled by typical molecular potentials, such as the Lennard-Jones potential. This is supported by some results we have obtained using classical perturbation theory, which appear to agree with the indications given, since several years, by numerical computations¹⁵⁻²³.

Thus, we conclude that the most general theorem available in classical perturbation theory, strongly supports the intuition of Boltzmann of almost a century ago, and deserves serious consideration. In any case, the relevance of metaequilibrium states has been often pointed out. This is illustrated by the physics of spin temperatures, where the system considered, that of nuclear spins, appears to have temperatures quite different from the temperature of the supporting system, that is the lattice, and from the temperature of the surrounding radiation field, just because "the internal thermal equilibrium time of the elements of the system is short compared to the time during which appreciable energy is lost or gained from other systems"²⁴. In such cases, for example, the first time is of the order of 10^{-5} s, while the relaxation time to the lattice is often of the order of many minutes or even days. Analogous situations are also met in connection with the rotational and the vibrational degrees of freedom of molecules, where a different temperature is often reported for any degree of freedom²⁵.

For a discussion of other problems related to the questions mentioned above, see refs 26-29.

Note added in proof: A first result proving the applicability of Nekhoroshev's theorem in the thermodynamic limit for short-range interactions, at least in a special case, has been independently obtained by C. E. Wayne³⁰.

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Maximum entropy signal processing in practical NMR spectroscopy

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NMR spectroscopy is intrinsically insensitive, a frequently serious limitation especially in biochemical applications where sample size is limited and compounds may be too insoluble or unstable for data to be accumulated over long periods. Fourier transform (FT) NMR was developed by Ernst¹ to speed up the accumulation of useful data, dramatically improving the quality of spectra obtained in a given observing time by recording the free induction decay (FID) data directly in time, at the cost of requiring numerical processing. Ernst also proposed that more information could be obtained from the spectrum if the FID was multiplied by a suitable apodizing function before being Fourier transformed. For example (see ref. 2), an increase in sensitivity can result from the use of a matched filter¹, whereas an increase in resolution can be achieved by the use of gaussian multiplication^{1,3}, application of sine bells^{4–8} or convolution difference⁹. These methods are now used routinely in NMR data processing. The maximum entropy method (MEM)¹⁰ is theoretically capable of achieving simultaneous enhancement in both respects¹¹, and this has been borne out in practice in other fields where it has been applied. However, this technique requires relatively heavy computation. We describe here the first practical application of MEM to NMR, and we analyse ¹³C and ¹H NMR spectra of 2-vinyl pyridine. Compared with conventional spectra, MEM gives considerable suppression of noise, accompanied by significant resolution enhancement. Multiplets in the ¹H spectra are resolved better leading to improved visual clarity.

For a given FID, many spectra can be produced which are consistent with the data—too many for easy comprehension. Nevertheless, we must, in practice, select just a single spectrum. It has been shown^{12,13} that MEM is the only generally consistent selection procedure. We maximize the entropy (negative information content)

$$S = -\int f(\nu) \log f(\nu) d\nu \quad (f \text{ normalized to } \int f d\nu = 1)$$

of the spectrum $f(\nu)$ of oscillator number densities, and in this way can select the spectrum which has the minimum of structure and hence fewest artefacts. Note that the ' $f \log f$ ' formula used here differs from the ' $\int \log f(\nu) d\nu$ ' entropy form commonly used^{14–16} in maximum entropy time-series analysis. The latter form produces a spectrum which gives greatest flexibility (maximum entropy) to individual samples from a time-series constrained by autocorrelation data. In NMR by contrast, we observe the FID time-series directly, and we are interested in the spectrum $f(\nu)$ as an object in its own right. For both these reasons, we should use the $f \log f$ form¹⁷. NMR data analysis is akin to radio interferometry^{18,19}, where $f \log f$ is also appropri-

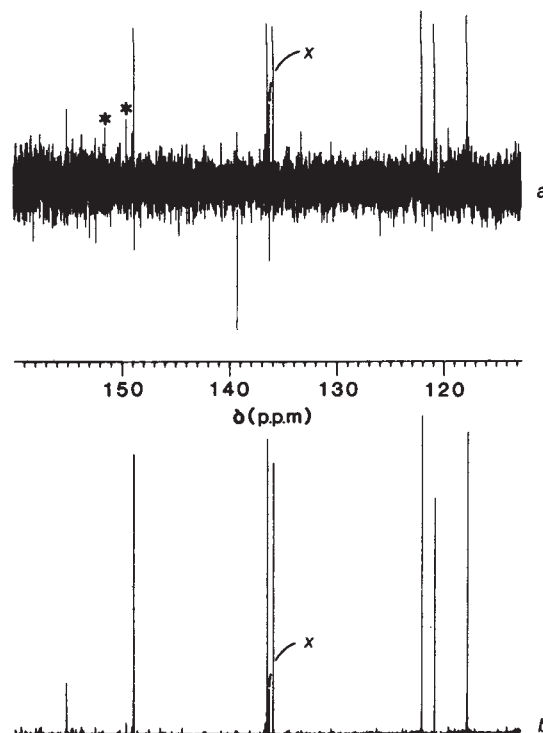


Fig. 1 a, Conventional Fourier transform of the ¹³C FID produced by a pulse of 1.6 μs on a sample of 2-vinyl pyridine in CDCl₃. b, MEM spectrum from the same FID. x, Instrumental artefact, attributed to imperfect transmitter gating.

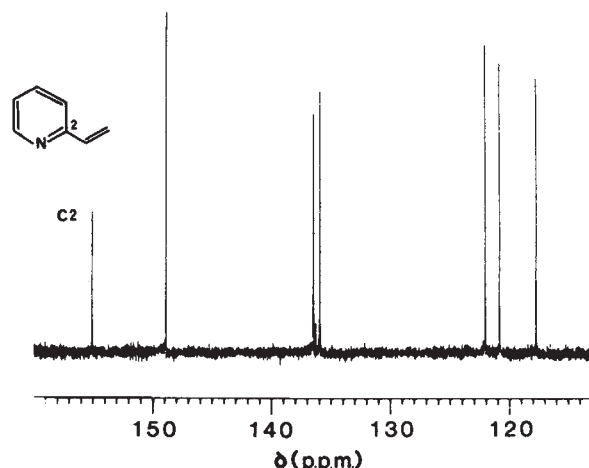


Fig. 2 Conventional Fourier transform of the ¹³C FID produced by a pulse of 13 μs.

ate, but NMR has the advantage of being free of peripheral arguments such as that about the role of the statistics of radio photons²⁰.

Note that MEM produces a spectrum which has to fit the data, in the sense that the distribution of oscillators would produce a FID not significantly different from the actual data. Conventional methods of processing, such as direct or apodized Fourier transforms, give spectra which lack this property.

The ¹³C data for 2-vinyl pyridine in CDCl₃ were recorded on a Bruker WM-250 instrument using the manufacturer's quadrature phase cycling and quadrature detection mode. We present results for a noisy test spectrum determined using a short pulse

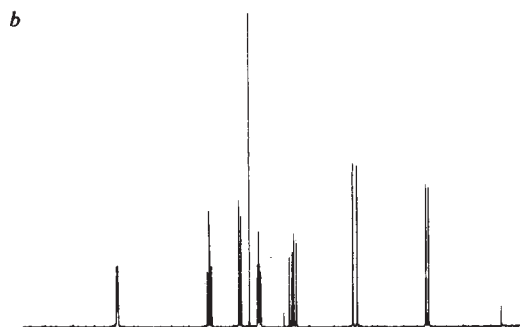
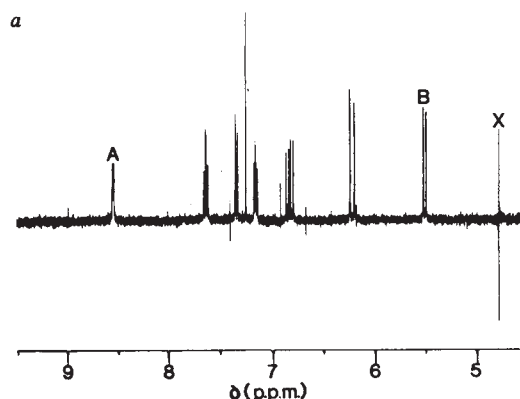


Fig. 3 a, ^1H spectrum of 2-vinyl pyridine produced by conventional Fourier transform. b, Corresponding MEM spectrum.

time (1.6 μs). We corrected the baseline of the FID and converted it from quadrature to cosine form²¹ over 8,192 points before zero filling to 16,384 points. The Cambridge MEM algorithm²² was used to find the two principal phase parameters, by maximizing the entropy over both the spectrum and these parameters simultaneously. The phase parameters were then used for both the conventional FT and MEM spectra shown in Fig. 1.

In the conventional FT spectrum, Fig. 1a, the signals assigned to C-3, C-4, C-5, C-6, C-7 and C-8 are reasonably clear of the noise, but the signal from C-2 is only marginally higher than some of the noise peaks (marked with asterisks). In the corresponding MEM spectrum obtained from the same data (Fig. 1b), noise is dramatically decreased, so that the peak for C-2 appears much more convincing. The reliability of the lines shown in the MEM spectrum can be assessed by comparison with a control FT spectrum (Fig. 2) produced from the same sample, using a longer pulse time (13 μs) to increase the signal-to-noise ratio. This proves that the C-2 line picked out by maximum entropy is a true signal, as opposed to the noise peaks marked with asterisks in Fig. 1a. The ability of MEM to discriminate in favour of real signals, and against noise of similar intensity, was reproduced in three other test spectra determined in the same conditions.

The ^1H data were recorded in a similar manner on a Bruker WH-400 instrument. We selected a moderately noisy spectrum to give a clear comparison between MEM and conventional procedures. Figure 3a shows the conventional Fourier spectrum, and Fig. 3b the corresponding MEM spectrum. In addition to the obvious suppression of noise, MEM has also sharply reduced the instrumental artefact, X. Two representative multiplets, A and B, are plotted on expanded scales in Fig. 4a (conventional) and Fig. 4b (MEM). The multiplets are appreciably sharper and better resolved in the MEM spectrum. Although not dramatic, this effect shows that suppression of noise is by no means accompanied by a loss of resolution, so that the technique should be of value in processing spectra that are crowded as well as noisy.

These results demonstrate that MEM provides a potentially important addition to the range of techniques currently available

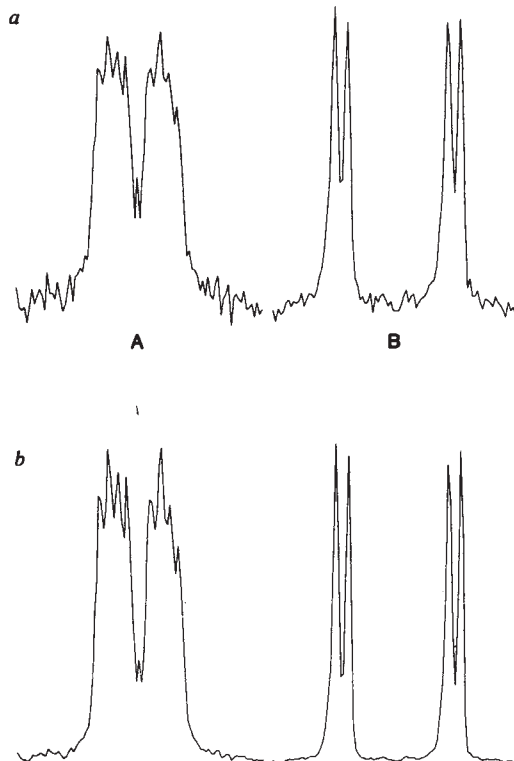


Fig. 4 a, Expansions of the multiplets A and B in the conventional spectrum (Fig. 3a). b, Expansions of the multiplets A and B in the MEM spectrum (Fig. 3b).

for producing NMR spectra. Details of the Cambridge MEM algorithm used to produce these spectra will be published elsewhere²². The disadvantage of MEM is the extra computing involved, amounting to about 1 min on a IBM3081 mainframe computer. The algorithm requires eight times the store and about 30 times the central processing time of an ordinary Fourier transform. Further work is in progress on the application of MEM to ^1H and ^2H NMR spectra, and also on the more sophisticated approach in which a two-dimensional map is reconstructed from one-dimensional data¹¹.

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High $^3\text{He}/^4\text{He}$ ratio in ocean sediments

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Two decades ago, Merrihue¹ reported $^3\text{He}/^4\text{He}$ ratios of $>10^{-4}$ in ferromagnetic separates from a Pacific deep ocean red clay and concluded that the high ratio is due to extraterrestrial debris amounting to $\sim 1\%$ of the sediment. A decade later Krylov *et al.*² compiled $^3\text{He}/^4\text{He}$ isotopic data on ocean sediments measured in the Soviet Union and observed that the $^3\text{He}/^4\text{He}$ ratio is generally higher in pelagic sediments where the sedimentation rate is lower. They suggested that the high $^3\text{He}/^4\text{He}$ ratio was attributable to extraterrestrial materials which were concentrated in slowly accumulating ocean floor. However, these important discoveries were almost completely neglected until we re-examined the problem. We have measured 39 sediments from 12 different sites, 10 sites from the western to central Pacific and two sites from the Atlantic Ocean. We find $^3\text{He}/^4\text{He}$ ratios $>5 \times 10^{-5}$ for six sites, well above the values generally observed in common terrestrial materials. The very high $^3\text{He}/^4\text{He}$ ratio in the sediments is probably due to input of extraterrestrial materials. Input of stratospheric dust of ≤ 1 p.p.m., which corresponds to a fallout rate of $\sim 2,000$ tons per year, can explain the observation.

All the sediments that we studied are piston-cored samples. While two Atlantic sediments (DSDP samples) are of Cretaceous age^{3,4}, all the Pacific samples are of Quaternary or, at most, of late Tertiary age^{5,6}. Petrographical descriptions and other data of the samples are given either in a DSDP Initial Report^{3,4} or in the Preliminary Report for the *Hakuho Maru* cruise^{5,6}. All the Pacific and Atlantic samples are very fine-grained typical pelagic sediment. A few hundred milligrams of a sample were hand-picked from a sediment core (diameter, 5 cm), which had been stored with a thick plastic cover (Pacific samples). Sediments were almost completely dried out and consolidated. Before gas extraction, samples were heated in vacuum at $\sim 150^\circ\text{C}$ to reduce surface air contamination. No further treatment of the samples, such as cleaning was attempted.

The detailed experimental procedures for the gas extraction and the mass spectrometric analysis are described elsewhere⁷. Helium isotopic measurements were done with a resolution of 600 and ^3He peak was completely resolved from HD and H_2 . The measured $^3\text{He}/^4\text{He}$ isotopic ratio was calibrated against an air helium isotopic ratio, which was measured repeatedly during the sample runs. The mass spectrometer has a dual collector for simultaneous measurements of ^3He and ^4He . Neither the mass spectrometer nor the extraction system have been used previously to analyse extraterrestrial materials. With these precautions, we believe that the measured helium isotopic ratios are satisfactorily free from interference or contamination effects, or from mass discrimination.

Table 1 shows the experimental data. The principle uncertainty in the experimental results comes from the correction for the hot blank. We assigned 50% of the hot blank as a possible error, in addition to the analytical error in the mass spectrometry, the latter being generally much less than the former. Because the variation in the hot blank throughout the experimental period was $<10\%$, a possible error of 50% of the hot blank would be an overestimation. Except for Ar, there was too little of the other noble gases to allow reliable quantitative or isotopic analyses. In most cases the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio is significantly higher than the atmospheric ratio, that is 295.5.

Measurements made on samples taken from the same level in a core (~ 1 cm apart in the same level) (KH75-3-5-2: levels 100 and 860 cm) show considerable difference both in the He content and in the isotopic ratio. The above consideration of the error estimation suggests that this difference is primarily due to sample heterogeneity owing to the small size (~ 100 mg) used for the experiment.

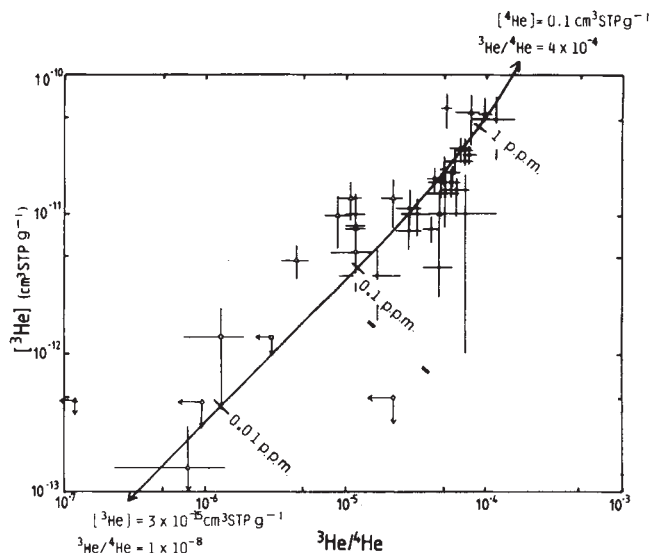


Fig. 1 ^3He content in sediments is plotted against $^3\text{He}/^4\text{He}$ ratio. The curve indicates the mixing between He in the cosmic dust ($^4\text{He} = 0.1 \text{ cm}^3 \text{ STP g}^{-1}$, $^3\text{He}/^4\text{He} = 4 \times 10^{-4}$) and in the sediments ($^3\text{He} = 3 \times 10^{-15} \text{ cm}^3 \text{ STP g}^{-1}$, $^3\text{He}/^4\text{He} = 10^{-8}$). Numbers on the curve indicate the degree of the contamination (in p.p.m.) of the cosmic dust in sediments. Δ , KH-80-3; $\circ$, KH68-4; $\bullet$, KH75-3-5; $\square$, KH-75-3-11; $\diamond$, DSDP.

Although the few data available make it difficult to draw a general conclusion on the distribution of $^3\text{He}/^4\text{He}$ ratios in ocean sediments, there appears to be no clear correlation between isotopic ratio and geography. However, it should be noted that high $^3\text{He}/^4\text{He}$ value ($>5 \times 10^{-5}$) is common in the Pacific sediments. Except for diamonds⁸, the highest $^3\text{He}/^4\text{He}$ ratio ever reported for terrestrial materials is 5.2×10^{-5} for Hawaiian olivine xenoliths⁹, the values for common terrestrial rocks such as granites being $<5 \times 10^{-5}$ (ref. 10). ^3He production in the Earth is practically negligible¹¹. It is also difficult to attribute the high $^3\text{He}/^4\text{He}$ ratio to some artificial nuclear fallout or debris, as samples were taken at more than two different levels in a core considerably deeper than the seabed and in several cases the deeper level showed the higher $^3\text{He}/^4\text{He}$ ratio. Because extraterrestrial materials have $^3\text{He}/^4\text{He}$ ratios generally higher than 10^{-4} with high helium content ($^4\text{He} \geq 10^{-5} \text{ cm}^3 \text{ STP g}^{-1}$)¹⁰, it is reasonable to assume input of some extraterrestrial materials to the sediments to account for their high $^3\text{He}/^4\text{He}$ ratios ($>5 \times 10^{-5}$). This assumption is supported by the following observation.

Figure 1 shows the $^3\text{He}/^4\text{He}$ ratio plotted against ^3He content. There appears to be a clear positive correlation between them which can be explained by the mixing of some materials with a high ^3He content and $^3\text{He}/^4\text{He}$ ratios with sediment which has a generally low He content¹². We can imagine either meteorite fragments or cosmic dust as possible contaminants. If meteorite fragments were produced by meteoritic impact on the Earth, the occurrence of high $^3\text{He}/^4\text{He}$ in ocean sediment must be sporadic. The observation of a common high $^3\text{He}/^4\text{He}$ ratio in ocean sediments, not only in the lateral but also in the vertical sections, contradicts the sporadic meteorite impact theory. Also, an impact would result in severe helium loss from the meteorite debris. Hence, we favour the cosmic dust origin for the anomalously-high $^3\text{He}/^4\text{He}$ ratio in the sediments.

If global fallout of cosmic dust were responsible for the high $^3\text{He}/^4\text{He}$ ratio as well as to the high ^3He content, we would expect an inverse relationship between the concentration of the cosmic dust and the sedimentation rate, because in the high accumulating region, the fallout cosmic dust must be diluted by terrestrial materials. Figure 2 shows the ^3He content plotted against the sedimentation rate (taken from refs 3–6). Two Atlan-

Table 1 Experimental data

Sample*	Locality (water depth)	Weight (g)	Hot blank		Sediments		
			^3He (10^{-11} $\text{cm}^3\text{STP g}^{-1}$)	^4He (10^{-7} $\text{cm}^3\text{STP g}^{-1}$)	^3He ($10^{-11}\text{cm}^3\text{STP g}^{-1}$)	^4He ($10^{-7}\text{cm}^3\text{STP g}^{-1}$)	$^3\text{He}/^4\text{He}$ (10^{-6})
KH68-4-15	11°59'N						
220–222.5 cm	169°58'W	0.131	0.07	0.42	5.8 ± 1.6	10.8 ± 2.0	53 ± 5
520–522.5 cm	(5,050 m)	0.116	0.07	0.62	1.0 ± 0.4	8.2 ± 1.3	12 ± 2
790–792.5 cm		0.131	0.05	0.53	0.8 ± 0.3	7.0 ± 1.4	12 ± 2
KH68-4-18	1°60'N						
203–205.5 cm	170°01'W	0.099	0.04	0.23	0.4 ± 0.3	0.8 ± 0.2	47 ± 21
506 cm	(5,470 m)	0.133	0.08	0.55	1.5 ± 0.6	2.4 ± 0.4	63 ± 15
979 cm		0.106	0.12	0.66	1.0 ± 0.9	1.4 ± 0.3	73 ± 49
KH68-4-20	2°28'S						
527 cm	170°00'W	0.117	0.10	0.61	1.4 ± 0.6	2.7 ± 0.4	52 ± 14
990 cm	(5,130 m)	0.106	0.08	0.48	1.1 ± 0.5	1.9 ± 0.3	58 ± 17
KH75-3-5-2	26°01'N						
4–5 cm	150°00'E	0.110	0.10	0.58	0.4 ± 0.2	2.1 ± 0.1	17 ± 8
100–101 cm (a)	(5,850 m)	0.135	0.04	0.42	1.8 ± 0.4	4.1 ± 0.3	44 ± 7
100–101 (b)		0.151	0.05	0.32	1.1 ± 0.4	3.7 ± 0.7	29 ± 5
205–206 cm		0.150	0.05	0.45	0.8 ± 0.2	2.7 ± 0.2	29 ± 6
296–298 cm		0.157	0.03	0.34	0.8 ± 0.2	2.5 ± 0.2	32 ± 6
378–379 cm		0.118	0.06	0.58	1.7 ± 0.4	3.4 ± 0.2	49 ± 9
408–411 cm		0.0296	0.19	0.82	0.5 ± 0.2	1.8 ± 0.8	30 ± 22
418 cm		0.121	0.05	0.54	1.0 ± 0.3	3.0 ± 0.2	33 ± 8
513–517 cm		0.138	0.04	0.44	3.0 ± 0.6	4.1 ± 0.2	72 ± 10
612 cm		0.144	0.04	0.38	1.7 ± 0.4	2.9 ± 0.2	58 ± 10
620–623 cm		0.135	0.03	0.23	1.5 ± 0.3	1.9 ± 0.2	78 ± 8
713–717 cm		0.142	0.06	0.53	2.1 ± 0.5	4.2 ± 0.3	51 ± 8
813–815 cm		0.136	0.06	0.47	2.4 ± 0.5	3.9 ± 0.2	61 ± 9
859–862 cm (a)		0.118	0.06	0.48	3.0 ± 1.1	4.4 ± 0.8	67 ± 12
859–862 cm (b)		0.0335	0.14	0.89	3.1 ± 1.5	2.6 ± 0.3	119 ± 43
890–891 cm		0.149	0.04	0.39	2.4 ± 0.4	3.4 ± 0.1	72 ± 10
KH75-3-11-2	32°59'N						
218–220 cm	153°29'E	0.0221	0.06	1.03	NM§	2.75 ± 0.92	<3
864–866 cm	(5,650 m)	0.133	0.04	0.62	0.13 ± 0.1	10.5 ± 2.2	1.3 ± 0.6
KH80-3-18	32°44.9'N						
397 cm	158°17.6'E	0.140	0.18	0.35	0.09 ± 0.09	3.1 ± 0.1	3 ± 3
529–531 cm	(2,640 m)	0.140	0.07	0.56	0.53 ± 0.25	4.4 ± 0.8	12 ± 4
KH80-3-22	31°16.2'N						
403 cm	153°42.9'E	0.128	0.05	0.52	0.47 ± 0.13	10.4 ± 0.7	5 ± 1
500–501 cm	(5,750 m)	0.144	0.06	0.45	0.95 ± 0.40	10.8 ± 2.3	9 ± 2
KH80-3-22	13°44.3'N						
200–201 cm	148°37.9'E	0.118	0.06	0.77	0.79 ± 0.27	6.7 ± 1.1	12 ± 2
550–552 cm	(5,800 m)	0.118	0.08	0.60	5.2 ± 2.1	6.6 ± 1.3	79 ± 17
KH80-3-28	11°03.3'N						
200–201 cm	151°34.6'E	0.125	0.07	0.51	1.3 ± 0.4	12.3 ± 2.3	11 ± 2
600–601 cm	(5,800 m)	0.114	0.08	0.44	1.3 ± 0.5	6.0 ± 1.0	22 ± 4
1,049–1,051 cm		0.141	0.6	0.49	5.3 ± 1.7	5.1 ± 0.9	105 ± 14
KH80-3-30	9°50.6'N						
999–1,003 cm	153°13.5'E	0.122	0.08	0.56	1.0 ± 0.5	2.1 ± 0.4	47 ± 15
	(5,480 m)						
Leg 43-386-42-3	31°11.21'N						
30–31 cm	64°14.94'W	0.223	0.0039	0.054	0.0065 ± 0.0158	0.099 ± 0.011	7 ± 15
109–110 cm	(4,782 m)	0.174	0.005	0.16	0.009 ± 0.008	1.9 ± 0.1	0.47 ± 0.44
Leg 43-387-30-1	32°19.2'N						
140–141 cm	67°40.0'W	0.197	0.015	0.14	NM§	NM§	<0.3
	(5,117 m)						

* About 100 mg of sample was handpicked from a sediment core at the designated interval (depth from the water bottom for the Pacific samples and depth at respective core section for the Atlantic samples).

† $^3\text{He}/^4\text{He}$ ratio is calibrated against air helium $^3\text{He}/^4\text{He}$ ratio to reduce a mass fractionation effect.

‡ Errors in ^3He and ^4He consist of 50% of the hot blank and 1σ in the mass spectrometry, the former being much larger than the latter.

§ A fraction of the extracted gas was introduced into a mass spectrometer. Hence, only the isotopic ratio was measured and the helium amounts were not measured (NM).

tic samples are quite isolated from Pacific samples. This may be due to helium-loss from the Atlantic samples, because they are older by a factor of 100 than the Pacific sediments. Ignoring the Atlantic samples, there still seems to be an approximately inverse relation between the ^3He content and the sedimentation rate. If we assume uniform fallout rate of cosmic dust and also that the initial ^3He content in sediment is negligible, then the ^3He concentration in the sediment, which can be taken as a

measure of the amount of cosmic dust input, can be expressed as a function of sedimentation rate (r) and flux of the cosmic dust fallout (f),

$$(^3\text{He})_{\text{sed}} = \frac{\alpha f}{\rho r} \quad (1)$$

where α and ρ denote respectively the concentration of ^3He in the extraterrestrial material and density of the sediment. For the

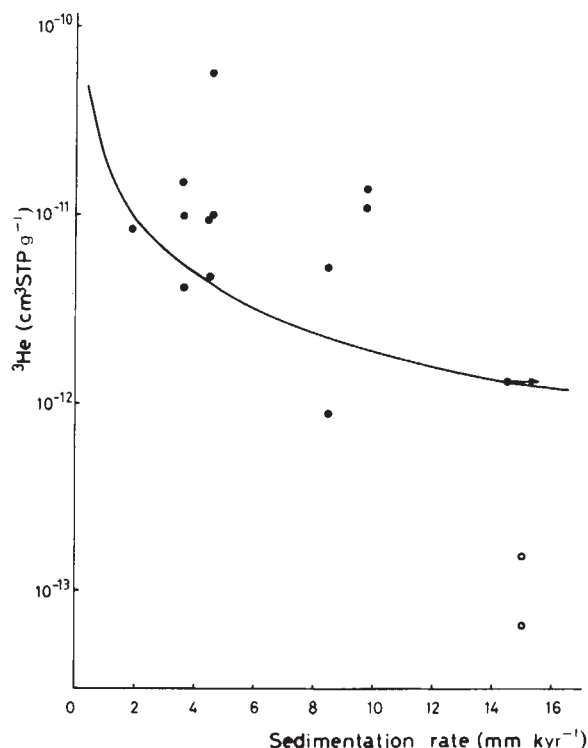


Fig. 2 ^3He content plotted against sedimentation rate. ○, The Atlantic DSDP samples which are of Cretaceous age. The Pacific samples (●) show an approximately inverse relationship between ^3He content and sedimentation rate. The curve is constructed by assuming uniform fallout rate (2,000 tons yr^{-1}) of cosmic dust ($^4\text{He} = 0.1 \text{ cm}^3\text{STP g}^{-1}$, $^3\text{He}/^4\text{He} = 4 \times 10^{-4}$).

^3He concentration in the cosmic dust, we take the value measured for stratospheric cosmic dust ($\sim 10\text{-}\mu\text{m}$ diameter size), that is $\alpha = 4 \times 10^{-5} \text{ cm}^3\text{STP g}^{-1}$. Putting $\alpha = 4 \times 10^{-5} \text{ cm}^3\text{STP g}^{-1}$ and $\rho = 2 \text{ g cm}^{-3}$ in equation (1), we can construct a family of curves corresponding to different values of f . Figure 2 shows a curve corresponding to $f = 4 \times 10^{-10} \text{ g cm}^{-2} \text{ yr}^{-1}$, which approximates the data. The value $f (= 4 \times 10^{-10} \text{ g cm}^{-2} \text{ yr}^{-1})$ corresponds to a cosmic dust fallout rate of about 2,000 tons yr^{-1} . The value may be favourably compared with the estimate made on satellite, radio and visual observations of about a few hundred tons per year for particle size below $10 \mu\text{m}$ in diameter¹³, considering the large uncertainty in the estimates of the fallout rate and the helium content of the cosmic dusts. Cosmic dust which has a diameter greater than $10 \mu\text{m}$ may not be important in the helium inventory of sediments, because the larger grains are likely to lose helium due to atmospheric impact heating¹⁴. Because various factors such as the non-uniform fallout rate of the cosmic dust, heterogeneity in their ^3He content and He-loss from the cosmic dust particles all act to disturb the simple inverse relation between ^3He content and sedimentation rate, it is quite normal to observe only an approximate inverse relation if the cosmic dust fallout is indeed responsible for the high $^3\text{He}/^4\text{He}$ ratio. Hence, we believe that it is the approximate inverse relationship and not the random relationship between the ^3He content and sedimentation rate which is significant and that this relationship is attributable to the global cosmic dust fallout.

Assuming that the cosmic dust is responsible for the high $^3\text{He}/^4\text{He}$ ratio, Fig. 1 also shows a mixing curve between the cosmic dust and the sediment. For the cosmic dust, we take $^4\text{He} = 0.1 \text{ cm}^3\text{STP g}^{-1}$ (ref. 14) and assume solar-wind implanted helium, that is, $^3\text{He}/^4\text{He} = 4 \times 10^{-4}$. For the uncontaminated sediments, we assumed a typical crustal value of 10^{-8} for $^3\text{He}/^4\text{He}$ ratio¹¹ and $^4\text{He} = 3 \times 10^{-7} \text{ cm}^3\text{STP g}^{-1}$ (ref. 12). The choice of the values for the sediments is not crucial. In fact, changing the value by a factor of 100 does not significantly alter

the mixing curve. Figure 1 shows that $<1 \text{ p.p.m.}$ input of the cosmic dust can account for the observed high $^3\text{He}/^4\text{He}$ as well as the high ^3He content. A preliminary study to search for extraterrestrial materials was made on sample KH75-3-5-2. Microscopic examination did not show any such evidence. Neutron activation analyses showed slightly higher Ni (200–600 p.p.m.) and Co contents (100–200 p.p.m.), but Cr (30–50 p.p.m.) is slightly lower than that observed in common crustal rocks. We could not detect Ir. Note that because of the very significant difference in He isotopic ratios of extraterrestrial and terrestrial materials, the method is potentially very useful for identifying input of the former to ocean sediments.

In the case of sample KH75-3-5-2 (total core length $\approx 9 \text{ m}$), there seems to be a progressive increase both in the ^3He content and $^3\text{He}/^4\text{He}$ ratio with increasing depth. This may be due either to the change in the cosmic dust fallout rate, or to that in the sedimentation rate, or to the both.

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Occurrence of subtidal dolomite in a hypersaline lagoon, Kuwait

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We report here the discovery of subtidal dolomite in a shallow hypersaline lagoon during a study of Recent carbonate and evaporite sediments in the Al-Khiran region of southern Kuwait in the northern part of the Arabian Gulf (Fig. 1). Diagenetic dolomites are known from a variety of geographical locations and a spectrum of environmental conditions^{1–6}. Although many occurrences of subaqueous dolomite have been documented^{1–4}, none has been reported from the subtidal areas of extensive Recent marine carbonate environments. The Al-Khiran dolomites show complex polyhedral crystal habits and a composition which ranges from $\text{Ca}_{51}\text{Mg}_{49}$ to $\text{Ca}_{56}\text{Mg}_{44}$. We postulate that a partial removal of dissolved sulphate ions (by bacterial reduction) from the interstitial waters of the sediments, the moderate $\text{Mg}^{2+}/\text{Ca}^{2+}$ ratios of the hypersaline waters and the occasional near-schizohaline conditions in the lagoon may be responsible for the formation of this dolomite.

The small Al-Khiran lagoon is the remnant of a much larger body of water which is now partly infilled and dry (Fig. 1), and is connected to the Arabian Gulf by a small tidal channel. The maximum spring tidal range is $\sim 2.2 \text{ m}$ and the lagoon drains out almost completely only two or three times each year. Most of the time there is at least 0.50 m of water in the lagoon, and its floor is colonized by a sparse cover of *Halodule* plants. Lagoon salinities are fairly uniform throughout most of the year (42–44‰) and occasionally rise to 50–52‰ in the hottest months (July–August). The average summer and winter air temperatures

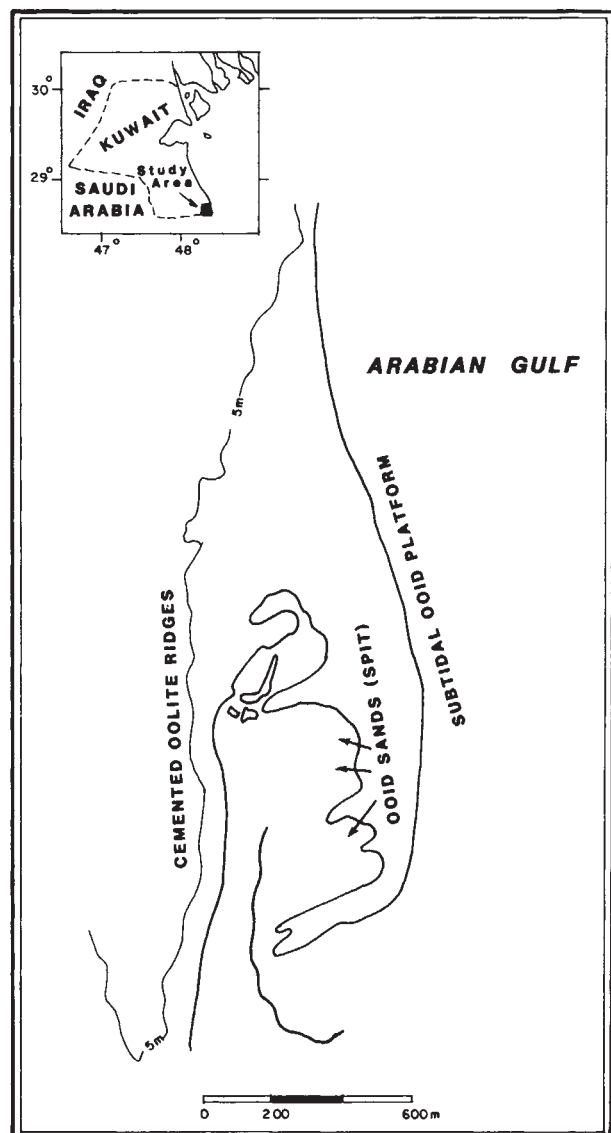


Fig. 1 The Al-Khiran lagoon and location of the study area.

are 45–47 °C and 12–15 °C, respectively. Water temperatures may exceed 30 °C in the summer and fall to as low as 8 °C in the winter months. Rainfall averages 100–120 mm yr⁻¹, but ranges up to 300 mm yr⁻¹.

The lagoon sediments are mostly a mixture of pellet muds and biodegraded skeletal material with some ooids (5–8%) and average ~70% aragonite. A representative lithological section (from cores) is shown in Fig. 2. The entire sediment column to a depth of more than 100 cm is highly reducing and organic-rich. Many samples from the lagoon bottom were analysed by X-ray diffraction and all contained diagenetic dolomite; it is most common in the upper 5 cm of the cores but occasionally is found at depths of 8–10 cm. The average dolomite content is 3.6% by weight of the total carbonate fraction and ranges from 3.2 to 4% (as determined by recalculation from 12 chemical analyses; the margin of error is too high when determining low dolomite contents by X-ray methods). The molar composition of dolomite was determined by measuring the peak shift of the (104) dolomite reflection relative to the (101) quartz reflection (internal standard)⁷; the standard error of the mean is ± 0.20 mol% CaCO₃ in dolomite for seven replicates. Dolomite composition averages Ca_{51.8}Mg_{48.2} (range is Ca₅₁ to Ca₅₆, based on 22 analyses). The dolomites are all Ca-rich and poorly ordered and have diffraction characteristics typical of naturally occurring early diagenetic 'dolomites'^{7,8}.

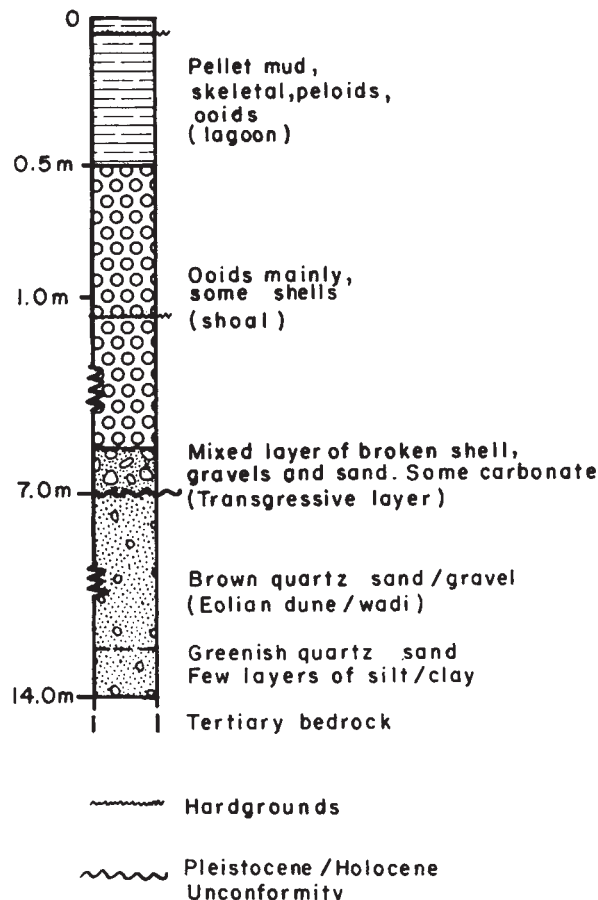


Fig. 2 Representative lithological section of the lagoon showing stratigraphical framework and depositional environments.

Scanning electron microscope (SEM) examination of several of these samples reveals that the dolomite crystals are well developed with complex polyhedral crystal habits, and range in size from $<1 \mu\text{m}$ to $\sim 5 \mu\text{m}$ (Fig. 3). They are found most commonly as clusters within minute 'solution' cavities on the surfaces of pellets and, less frequently, within the aragonitic needle mud fraction. Surprisingly, the development of the unit rhomb alone is very rare. The most common crystal form is the unit rhomb bevelled by a pair of triangular-shaped basal pinacoids (0001; 000 $\bar{1}$) which may be equally or subequally (asymmetrically) developed in a given crystal (Fig. 3). Less commonly, various other vicinal facets develop, modifying the rhombic symmetry. Although X-ray analysis does not indicate the presence of any gypsum, the scanning electron micrographs do show trace quantities of microcrystalline, euhedral gypsum crystals in the sediment.

The concentrations of Mg and Ca in lagoon surface and squeezed interstitial waters were determined by standard atomic absorption methods and sulphate concentration was measured by a turbidimetric method⁹. Carbonate alkalinity was determined by acid titration. All analyses were performed with reference to standard seawater to ensure good precision and accuracy. The results are summarized in Table 1.

The data indicate moderate Mg/Ca ratios, but more important from the point of view of dolomite formation is the reduced levels of dissolved sulphate in the pore fluids compared with surface waters; this is due to the high degree of microbial sulphate reduction within the sediments which prevents large-scale build-up of SO₄²⁻ concentrations. The alkalinity of the reduced pore waters is slightly higher than that of surface waters, but we do not consider this significant, particularly because there was a lapse of ~ 30 h between collection and analysis (most critical for alkalinity).

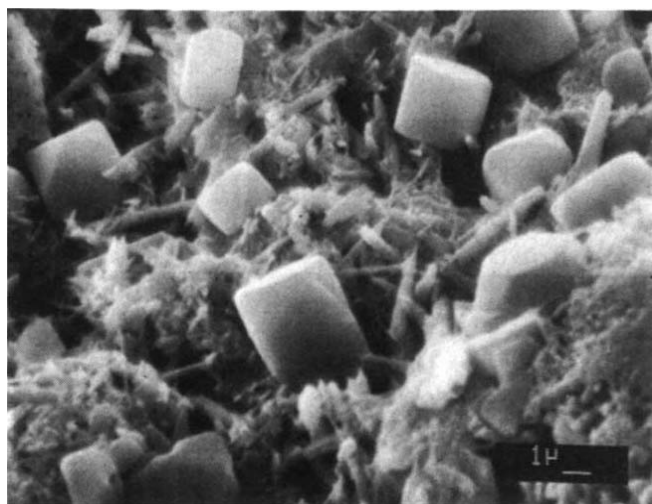


Fig. 3 Scanning electron micrograph of subtidal dolomite crystals from the Al-Khiran lagoon. The sample is from 7 cm depth in the core. Note the polyhedral crystal habits and particularly the unit rhomb modified by basal pinacoids.

In the winter months of 1982–83, ~240 mm of rainfall were recorded in the area, with one thunderstorm accounting for 128 mm in 18 h. The latter episode diluted and flushed the lagoon waters temporarily, but significantly, bringing it down to ~16‰ salinity (and possibly much lower as salinity was measured the day after the thunderstorm subsided) and producing mild schizohaline conditions. Freshwater flushing of hypersaline environments can be an important process in dolomite formation¹⁰. Freshwater flushing, however, does not markedly reduce the Mg/Ca ratio of the lagoon waters from its normal hypersaline values; if this were the case, the reduction of sulphate ion levels in the sediments (through microbial activity and/or by producing schizohaline conditions) could be more important than high Mg/Ca ratios or high salinities for dolomite formation¹¹. Baker and Kastner suggest¹¹ that high concentrations of SO_4^{2-} ions suppress efficient dolomite nucleation in subtidal or open marine environments, and may be one of the kinetic barriers preventing large-scale dolomite precipitation from seawater, despite the fact that it is the thermodynamically preferred carbonate phase.

Based on the vast amount of data already available, it seems that high Mg/Ca ratios and salinities of pore waters should generate dolomites that are near stoichiometric in composition, and as the Mg/Ca ratio and salinities decrease the dolomites should depart from ideal stoichiometry⁶. The Al-Khiran data seem to agree with this general premise. However, other factors may be involved in determining stoichiometric behaviour⁶.

The presence of dolomite in the top few centimetres of the sediments indicates that it formed in the very recent past of the lagoon and with the advent of quiet water conditions (pellet muds). The ooid sands below the dolomite represent the open and high-energy conditions of a shoal environment. Furthermore, the presence of patchy subtidal hardgrounds would act as a barrier for flushing and downward movement of potentially dolomitizing fluids. This difference between the nature of the lagoon now compared with earlier times shows the importance of the environment in dolomite genesis.

Dolomitization is a very complex process and opinion is divided as to the mechanisms involved in formation of diagenetic dolomites^{2,3,4,6,10,11}. What is the mechanism operating during Recent dolomite genesis in an environment of reduced sulphate concentration and moderate Mg/Ca ratios, with the possible influence of occasional schizohaline conditions in the lagoon? Mass balance calculations indicate that with Mg/Ca ratios of 5.2–6.3 and average porosities in similar sediments, more dolomite should form than is observed in Al-Khiran. Massive dolomitization, however, (even in sabkhas) depends on more complex hydrodynamic behaviour of pore fluids in a sediment

Table 1 Carbonate alkalinity and levels of SO_4^{2-} , Ca and Mg in lagoon surface and interstitial waters

	Surface waters	Reduced pore waters
SO_4^{2-} concentration (M)	0.030–0.036 (0.034)	0.020–0.028 (0.025)
Carbonate alkalinity (M)	0.0033–0.0034 (0.0033)	0.0036–0.0041 (0.0037)
Mg ²⁺ /Ca ²⁺ ratio (molar)	5.2–6.3	5.4–6.2

Values are the range (mean).

column (Al-Khiran sabkhas show Mg/Ca values of 10–14, yet the dolomite levels are rarely above 8%). Hence, the Mg/Ca ratio argument appears to be a geochemically weak one. However, moderate Mg/Ca values may be essential to stabilize the dolomite once it is formed⁴. Thus, the low SO_4^{2-} ion concentrations in the pore fluids are the most likely controlling factor in dolomite genesis. In fact, the effects of sulphate reduction on carbonate alkalinity are regarded by some authors as a critical factor for carbonate precipitation^{4,11}. We do not imply that the dolomite is a direct precipitate from lagoon waters; rather, the textural evidence from scanning electron microscopy demonstrates that it may be a replacive, cement phase, having formed at the expense of micritic aragonite (Fig. 3).

It is instructive to compare Al-Khiran with other geochemically similar environments of Recent dolomite formation. Subtidal dolomite has not been reported from the Trucial Coast lagoons of the southern Arabian Gulf^{12,13}, where schizohaline conditions are probably never achieved (only 30–40 mm rainfall yr^{-1}). In contrast, Al-Khiran experiences slight drops in salinity every year during the wet season and mild schizohaline conditions at least once every few years, particularly during neap-tidal phases when the volume of water in the lagoon is considerably reduced. In the extremely evaporitic and stratified Solar Lake⁴, dolomite formation is seasonal and the driving mechanism is the very efficient seepage-reflux of Mg-rich hot brines, which also explains why it forms at depth and not at the surface of the sediments. The dolomite here too is a replacement following aragonite. The occurrence of dolomite in Naxos³ represents an example of dolomitization in much milder environmental conditions. These two cases and Al-Khiran have one common geochemical feature, that is, the reduction in sulphate ion levels of the potentially dolomitizing fluids, with a dramatic increase in carbonate alkalinity in the case of the Solar Lake⁴. Thus, a combination of water dynamics and fluid compositions could account for these occurrences.

An important implication of this study is that in a prograding coastline, there could be two generations of dolomite in the sediments, having formed either during the subtidal lagoonal phase and/or during the later supratidal sabkha phase by flood-recharge and refluxing, and it may not be possible to distinguish between them. We hope that such dolomite occurrences will encourage carbonate geologists to reinterpret at least some of the literature on dolomite.

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Cobalt in pore waters of marine sediments

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During early diagenesis on the sea floor, metal-recycling processes exert important controls on the preservation rates and distributions of metals in marine sediments. The elucidation of internal recycling processes, therefore, is important in understanding trace-metal mass balances in the oceans. Recent measurements of cobalt in seawater¹ suggest that cobalt is scavenged from the deep sea and hence follows biogeochemical pathways similar to manganese. We present here measurements of cobalt in pore waters of marine sediments and find that cobalt was released into pore waters in the sub-oxic sediment zone during dissolution of sedimentary manganese oxides (Table 1, Fig. 1). Solid-phase cobalt concentrations in surface (oxic) sediments, above the pore water remobilization horizon, are almost double those measured in the deep (anoxic) sediment zone (Table 2, Fig. 1). The sediment and pore water distributions indicate that a large fraction of sedimentary cobalt is recycled with manganese between oxic and sub-oxic pore waters and redeposited in surface (oxic) sediments.

Pore water samples were obtained from the Hatteras Abyssal Plain and Continental Rise sites of the western Atlantic and from the San Clemente Basin off southern California (Table 1). Sediments from gravity and box cores were loaded into polyethylene tubes and pore waters separated by centrifuging at 13,000 r.p.m. for 5 min (20,800g). Pore waters were filtered through pre-cleaned 0.4- μ m polycarbonate filters and acidified to pH ~2 in pre-cleaned polyethylene vials. Cobalt was determined by direct-injection atomic absorption spectrometry on a Perkin Elmer 5000 with Zeeman background correction capability². The detection limit for 60 μ l injections was ~2.5 nmol kg⁻¹ and precision is $\pm$ 10% at concentrations of 10–50 nmol kg⁻¹. The technique is not sensitive enough to detect the cobalt levels measured in seawater¹.

The concentrations of cobalt, manganese, nitrate and ammonia in pore waters are listed in Table 1. We use nitrate and ammonia data to infer the 'redox' character of the sediments³. The depth of the nitrate maximum indicates the approximate depth of penetration of bottom water oxygen into the sediments⁴ and, therefore, oxic sediments. Nitrate depletions below the maximum measured, and the appearance of

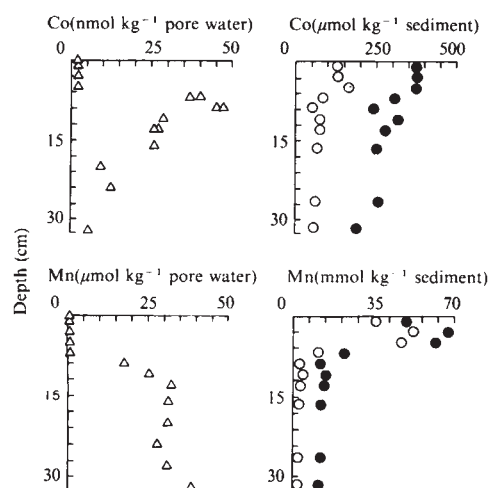


Fig. 1 Co and Mn pore water and sediment concentrations from Hatteras Continental Rise, core BC-4. ○, Leach; ●, total.

Table 1 Pore water concentrations

Depth (cm)	NO ₃ (μmol kg ⁻¹)	NH ₃ (μmol kg ⁻¹)	Mn (μmol kg ⁻¹)	Co (nmol kg ⁻¹)
HAP SC3; 32°34.0' N, 69°49.7' W; depth 5,330 m				
BW	22.5	0.9	<0.5	<2.5
0-2	24.6	9.3	<0.5	<2.5
4-6	21.7	3.4	<0.5	<2.5
8-10	32.5	6.5	1.6	18
12-14	33.3	8	<0.5	<2.5
16-18	35.4	12.2	<0.5	<2.5
25-30	35.6	5.7	<0.5	<2.5
35-40	34.5	8	<0.5	<2.5
50-55	32.3	4.5	1.1	<2.5
HAP PC11; 32°29.2' N, 70°59.9' W; depth 5,330 m				
BW	22.9	1.9	<0.5	<2.5
0-3	28.4	4.7	<0.5	<2.5
3-5	28.7	5.1	<0.5	<2.5
5-10	28.7	3.4	<0.5	<2.5
15-20	29.4	2.4	<0.5	<2.5
30-35	26.4	3.8	<0.5	<2.5
40-45	23	4	<0.5	<2.5
50-55	20.9	3.3	<0.5	<2.5
60-65	17.5	3.2	<0.5	<2.5
80-85	8.1	4.3	<0.5	<2.5
HCR BC4; 36°21.4' N, 71°34.2' W; depth 4,175 m				
BW	21.6	0.5	<0.5	<2.5
0-2	30.9	8.1	<0.5	<2.5
2-4	30.7	3.9	<0.5	<2.5
4-6	22.5	3.6	<0.5	<2.5
6-8	8.3	3.1	0.8	37.1
8-10	3.5	6.5	17.8	40.2
10-12	3.5	11.7	25.6	47.3
12-14	3.5	16.5	32.5	45.4
14-18	3.9	21.2	31.6	28.8
18-22	3.9	40.8	31.4	26.7
22-26	2.9	—	28.3	26
26-30	4.4	62.0	31.4	26
30-34	3.7	—	38.7	9.3
HCR GC5; 36°19.8' N, 71°27.1' W; depth 4,200 m				
BW	20.8	0.5	<0.5	<2.5
0-2	19.9	4.8	<1	<2.5
2-4	3.3	4.2	6	8.9
4-6	2.5	8.6	17.3	24.6
6-8	2.2	13.5	20.7	22.4
8-10	2.5	17.4	21.2	20.8
15-17	0.5	39	19.7	12.4
30-32	0.5	54.6	19.7	13.6
45-47	0.5	81.4	16.7	7.6
63-65	0.5	97	17.3	5.6
78-80	0.5	106	16.1	3.8
93-95	0.5	146.2	17	<2.5
106-108	0.5	159.5	20.2	<2.5
121-123	0.5	195.6	14.2	<2.5
148-150	0.5	227.6	15.9	<2.5
163-165	0.5	252.7	15.2	<2.5
178-180	0.5	275.6	13.1	<2.5
SCB-2; 32°33.1' N, 118°0.8.1' W; depth 1,930 m				
BW	42.3		0.01	<2.5
0.6	0.2		7.4	<2.5
1.9	0.2	No data	39.6	7
5	0.2		85.2	9.1
6.6	0.2		117.9	15.1
8.2	0.2		129.5	12.4
11.2			171.9	10
14			123.2	7.1
17.5			105.2	7.2

HAP, Hatteras Abyssal Plain; HCR, Hatteras Continental Rise; SCB, San Clemente Basin. Reported Co and Mn Atlantic bottom water (BW) concentrations do not reflect 'true' bottom waters. However, we emphasize that we are not able to distinguish differences between bottom waters and surface sediment (oxic) pore water concentrations. Co and Mn were measured in Atlantic Bottom waters (H. Windom, personal communication) at 20 pmol kg⁻¹ and <1 nmol kg⁻¹ respectively at depths of 3,800–5,600 m. We have measured the reported Mn content from San Clemente Basin of 10 nmol kg⁻¹ on several cruises to this area. Co has been measured¹ at 27 pmol kg⁻¹ in coastal Pacific waters at 1,900 m depth.

Table 2 Sediment concentrations

Depth (cm)	Total Mn (mmol kg ⁻¹ dry sediment)	Leach Mn (mmol kg ⁻¹ dry sediment)	Total Co (μmol kg ⁻¹ dry sediment)	Leach Co (μmol kg ⁻¹ dry sediment)
1	49.4	36.0	376.5	130.2
3	67.8	52.7	379.4	133.3
5	62.2	47.3	375.8	165.7
7	22.7	11.2	308.4	86.1
9	12.1	3.2	242.9	53.5
11	14.4	4.8	321.2	78.9
13	13.8	3.7	280.3	77.4
16.5	12.1	3.2	254.7	70.0
26.5	12.1	2.7	261.5	66.5
31.5	11.2	2.6	196.1	61.6

All data are corrected for % CaCO₃. A USGS rock standard GSP-1 and an in house Pacific pelagic sediment standard A3A were analysed along with BC-4 samples. Reported²⁰ Co and Mn concentrations for GSP-1 are 6 p.p.m. and 0.04 wt % Mn, respectively, compared with our analyses of 6.5 ± 1.0 p.p.m. Co and 0.03 wt % Mn. Similarly, reported concentrations of standard A3A (A. Graybeal, personal communication) are 66 ± 6 p.p.m. Co and 6.6 ± 0.1 wt % Mn, while our data are 58 ± 5 p.p.m. Co and 6.7 ± 0.1 wt % Mn.

manganese in the pore waters, indicate the sub-oxic zone of sediments³. Rapidly rising ammonia, produced during sulphate reduction^{3,5}, indicates anoxic sediments.

We find that pore water cobalt concentrations are undetectable (<2.5 nmol kg⁻¹) in oxic zone sediments of Hatteras Abyssal Plain cores PC-11 and SC-3 (Table 1). However, cobalt concentrations rise rapidly from these concentrations in the oxic zone to maximum concentrations of 25–50 nmol kg⁻¹ in the manganese reduction zone of Hatteras Continental Rise sediments BC-4 and GC-5. Pore water cobalt and manganese are shown for one core, BC-4, in Fig. 1. Similarly, in the San Clemente Basin pore water cobalt rises to 15 nmol kg⁻¹ in the manganese reduction zone. Cobalt has been found adsorbed onto the surfaces of manganese oxide phases^{6–8} and oxidized there to Co(III) (refs 9, 10). The data presented here indicate that cobalt is released to pore waters during reductive dissolution of manganese oxides. The shape of the cobalt profile indicates that cobalt in pore waters is diffused towards the sediment-seawater interface and is removed from pore waters to surface oxic sediments. The maximum cobalt concentration appears at shallower depths than the maximum manganese concentration, an unexpected result if cobalt and manganese undergo congruent dissolution. While we cannot explain this observation, cobalt in pore waters may diffuse further than manganese towards surface oxic sediments before it is removed to the solid phase. Cobalt concentrations decrease below the observed maximum in the sub-oxic sediment zone to near undetectable levels measured in anoxic sediments at depths <100 cm in Hatteras Continental Rise cores BC-4 (Fig. 1), and GC-5 (Table 1). Decreasing cobalt concentrations probably result from the precipitation of insoluble cobalt sulphides¹¹, formed during sulphate reduction which begins at shallow depths (~10 cm) in Hatteras Continental Rise cores.

Sediment samples for 'total' metal analyses were digested in an aqua-regia-hydrofluoric acid mixture^{12,13}. Extracts of weakly bound or leachable metals were prepared by shaking sediment samples in ammonium oxalate-oxalic acid¹⁴ mixtures. 'Total' metal digestions and 'leach' extracts were analysed for cobalt and manganese by flameless and flame atomic absorption spectrometry respectively. The analytical precision of 'total' and 'leach' cobalt analyses was respectively ±12% and ±5% over the concentration ranges reported here. 'Total' and 'leach' cobalt and manganese from the Hatteras Continental Rise core BC-4 are also shown in Fig. 1 and summarized in Table 2. Cobalt concentrations are highest in the top <7 cm of sediments and decrease sharply to near asymptotic concentrations at depths near 11 cm. Cobalt enrichments in the core tops are similar to the sediment manganese distributions (Fig. 1). However, while the top/bottom enrichment of sedimentary 'leach' manganese

Table 3 Comparisons of pore water cobalt and manganese fluxes with surface sediment excess metal accumulation rates, Hatteras Continental Rise, core BC-4

Core and metal HCR BC-4	Excess metal accumulation rate		
	Pore water flux (nmol cm ⁻² yr ⁻¹)	'Total' (nmol cm ⁻² yr ⁻¹)	'Leach' (nmol cm ⁻² yr ⁻¹)
Mn	160	113	109
Co	0.5	0.3	0.2

Pore water flux = $\phi D_s dc/dz$. The 'average' concentration gradient (dc/dz) between oxic and sub-oxic sediments has been estimated between depths 5–13 cm and 5–11 cm for Mn and Co respectively. ϕ is porosity (0.7) and D_s is the bulk sediment diffusion coefficient²¹ (D_s for Mn and Co are 57 and 63 cm² yr⁻¹ respectively) obtained from the ionic diffusion coefficient²² corrected to *in situ* temperatures (2°C) and further corrected for porosity and a formation resistivity factor²¹ of 2.5. We estimate a 50% uncertainty in the pore water flux calculations. 'Excess' metal accumulation rate = $\Delta C \times D \times S$. ΔC is the concentration difference between mean surface oxic zone (<7 cm) metal concentrations and asymptotic concentrations in anoxic zone sediments for both 'total' and 'leach' metal digestions and extracts respectively. The *in situ* dry bulk density (D) was estimated²³ at 0.32 and the sedimentation rate estimated from the down-core CaCO₃ profile²⁴, from core GC-5, at 10 cm kyr⁻¹.

is near 10-fold, that of cobalt is only near two-fold. The processes of manganese release to sub-oxic pore waters and reprecipitation in surface oxic sediments, resulting in an enriched zone in oxic sediments, have been well documented^{3,15–18}. The data presented here show that cobalt is recycled during dissolution and reprecipitation of sedimentary manganese oxides between sub-oxic and oxic sediments and pore waters. The recycling processes result in a surface sediment cobalt trap.

We now focus on the recycling between oxic and sub-oxic sediment zones and test the coherence of the pore water and sediment data shown in Fig. 1. First, we calculate the flux of dissolved metal diffusing upward from the cobalt remobilization zone in sub-oxic sediments to surface oxic sediments. Then, we compare this flux with the accumulation rate of 'excess' metal in surface sediments. 'Excess' metal is defined as the difference between the mean metal sediment content in the top 7 cm of oxic sediment and the asymptotic metal concentration in anoxic sediments (Table 2). The calculations are summarized in Table 3 and indicate that for both manganese and cobalt, the 'excess' accumulation rate of metal calculated from the 'leach' sediment data accounts for most 'excess' metal accumulation calculated from 'total' sediment concentrations. These 'excess' metal accumulation rate estimates are comparable with the upward flux of metal through pore water to surface oxic sediments, indicating that the recycling of metal between pore waters and surface sediments is in an apparent steady state and is a contemporary oceanographic feature. A measure of the extent of metal recycling between sediments and pore fluids is provided by comparing the dissolved manganese (160 nmol cm⁻² yr⁻¹) and cobalt (0.5 nmol cm⁻² yr⁻¹) fluxes upward through pore waters (Table 3) with the mean total manganese (160 nmol cm⁻² yr⁻¹) and cobalt (1.1 nmol cm⁻² yr⁻¹) sedimentation rates (mean total metal content $\times$ sedimentation rate $\times$ *in situ* dry density) in the top 7 cm of sediments. (Note the distinction between total mean sedimentation rate used here and 'excess' accumulation rate defined in Table 3.) These comparisons suggest that large fractions of the solid phase cobalt and the manganese settling into the remobilization zone are recycled through pore waters to surface oxic sediments at this rapidly sedimenting Continental Rise site.

During early diagenesis in sediments the behaviour of cobalt is, therefore, similar to that of nickel¹⁹. Both cobalt and nickel are released to pore waters in the manganese reduction zone and recycled between oxic and sub-oxic sediments and pore waters. This type of recycling is in contrast to that of copper where remineralization occurs primarily in the oxygen reduction

zone, and most copper is recycled between oxic sediments and pore waters and overlying deep oceanic waters¹⁹.

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Contribution of gaseous sulphur from salt marshes to the global sulphur cycle

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Salt marshes have high rates of sulphide production due to dissimilatory sulphate reduction^{1,2}; and because their anaerobic sediments are exposed to the atmosphere, they are thought to be important sites of biogenic sulphur emission^{3,4}. We report here a synthesis of the results of an annual set of monthly flux measurements of hydrogen sulphide (H₂S), dimethyl sulphide (DMS), carbonyl sulphide (COS), carbon disulphide (CS₂) and dimethyl disulphide (DMDS) from a *Spartina* marsh and an adjacent tidal creek. Dimethyl sulphide and H₂S were the predominant gases released from the *Spartina* marsh (49% and 35% of the total, respectively) while H₂S was the major gas (71%) emitted from the creek site. We suggest that the short-lived sulphur gases released from marshes may have an important role in local atmospheric sulphur budgets. Globally, saline marshes may release a total of 1.7 Tg S yr⁻¹ (1 Tg = 10¹² g) into the atmosphere, which includes more than double the quantity of two important long-lived sulphur gases, COS and CS₂, needed to sustain the stratospheric sulphate layer.

Gaseous sulphur flux measurements were made using a FEP Teflon flow-through chamber⁵ which covered the sediment or water surface. Ambient air was continuously drawn through the chamber at a velocity of 0.12 m s⁻¹ and the difference between the gaseous sulphur concentrations in the chamber input and output air was used to determine the net uptake or release of the sulphur gases. This chamber design allowed the sulphur gases in the incoming ambient air to interact with the grass, sediment, or water surfaces so that net fluxes (emission minus uptake) were measured.

The volatile sulphur compounds were collected in the field from the chamber input and output air streams with glass tube traps packed with the solid adsorbents Molecular Sieve 5A and Tenax GC⁶. The chamber air streams were sampled at 40–100 cm³ min⁻¹ for 1–2 h depending on the anticipated sulphur

concentrations for the sampling date. In the laboratory, the sulphur species were thermally desorbed from the trap, separated by temperature programming a Chromasil 330 column (10 foot × $\frac{1}{8}$ inch) and the amounts measured by a sulphur specific flame photometric detector⁶. Trap recoveries were determined with humid air (30–95% RH) to mimic field conditions.

We measured the gaseous sulphur fluxes during 1979 and 1980 from a short *Spartina alterniflora* marsh⁵ and from an adjacent tidal creek (our unpublished work) in Great Sippewissett Marsh, Falmouth, Massachusetts. The sulphur fluxes from the *Spartina* site were measured with the grass intact and the chamber sealed to the peat surface. The fluxes from the creek site were obtained with a chamber which either rested on the creek sediment or floated on the surface of the tidal water. Each sampling usually consisted of a series of 1-h flux measurements made continuously for two consecutive tidal cycles (26 h) to obtain a daily flux estimate. The samplings were done at least once a month from February through December at the marsh site and from May through December at the creek site. We assumed that there were negligible releases in January from the *Spartina* site. Emission rates from the creek site for March and April were assumed to be equal to the rate measured in May. The creek site was ice covered (15 cm) in January and February so that flux measurements were not possible. We assumed negligible releases for those two months. We did not structure the sampling design to examine specifically the effects of the diurnal or tidal cycles on the emissions, but we included a variety of tidal and day-night regimes to obtain unbiased annual flux estimates.

Dimethyl sulphide and H₂S were the major components released from the *Spartina* site while H₂S was the major compound released from the creek site (Table 1). We calculated the annual sulphur emissions from Great Sippewissett marsh by weighting equally the daily fluxes from the *Spartina* and creek sites and then extrapolating to monthly fluxes. Maximum emission of each of the individual sulphur gases did not coincide but occurred from July through October (Fig. 1). Emissions during the summer and autumn months (June through October) accounted for 73% of the total release. We estimated a total emission from the marsh of ~4.5 g S m⁻² yr⁻¹ with H₂S (48%) and DMS (33%) the predominant sulphur gases emitted.

As the major gases released from marshes are short-lived compounds, H₂S, DMS and DMDS, the rapid tropospheric oxidation of these gases might be expected to produce elevated atmospheric levels of SO₂ and SO₄ in the vicinity of marshes. This could be important in locations where marshes constitute a large fraction of the land area. For example, Reisinger and Crawford⁷ have hypothesized that marshes in the southern coastal US have an important role in the atmospheric sulphate concentrations in the Tennessee Valley.

Comparison of salt marsh sulphur fluxes with other biogenic sulphur sources (Table 2) indicates that marshes have the highest areal gaseous sulphur emissions with rates at least 10- to 100-fold greater than the emissions from oceans or inland soils. We estimated the inland soil emissions (Table 2) using the data reported by Adams *et al.*⁸ from an extensive survey of sulphur fluxes from eastern US soils. We combined the average measured fluxes, which included some seasonal measurements (April through November), from 29 locations for seven different non-swamp (aerobic) soil orders and took the mean values. We also

Table 1 Annual gaseous sulphur emissions from the *Spartina* and tidal creek sites (g S m⁻² yr⁻¹)

	<i>Spartina</i> site	Tidal creek
H ₂ S	2.05	2.32
DMS	2.87	0.16
COS	0.30	0.34
CS ₂	0.16	0.20
DMDS	0.42	0.25
Total	5.80	3.27

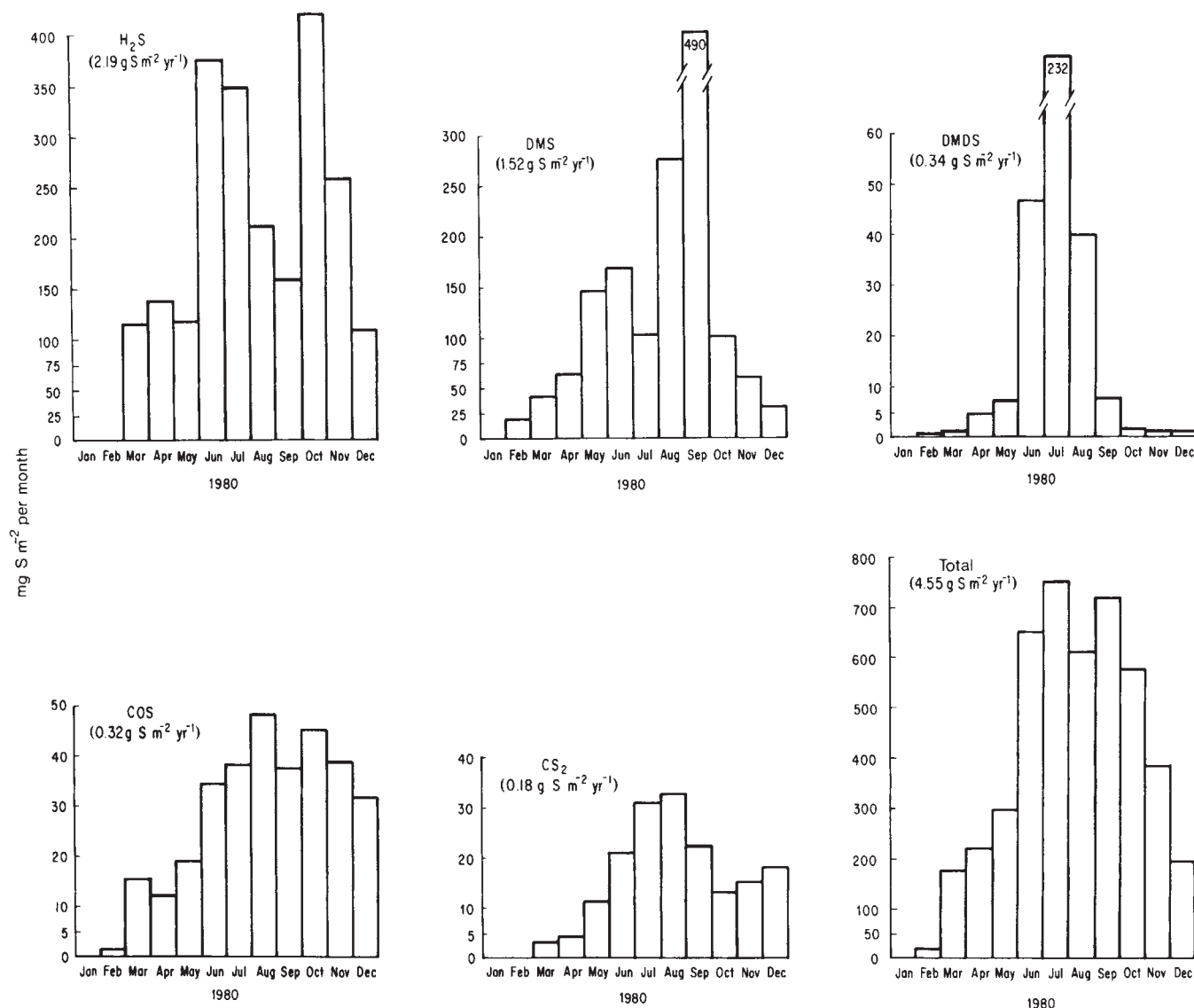


Fig. 1 Annual cycle of monthly fluxes for the individual sulphur gases and the total monthly release for Great Sippewissett Marsh. Note that the flux rate scales are different.

adjusted each of the mean annual fluxes by increasing them two-fold (D.F. Adams, personal communication) because the data reported for inland soils were believed to represent minimum values⁹.

A global estimate of the biogenic sulphur released from saline marshes can be calculated if we assume that: (1) all marshes have the same emission rates as Sippewissett Marsh; (2) the global marsh area¹⁰ is $3.8 \times 10^{11} \text{ m}^2$; (3) marshes are divided equally into vegetated areas and open-water areas of creeks and channels. We estimate that marshes release a total of 1.7 Tg S yr^{-1} to the troposphere of which 81% are the short-lived species H₂S (0.83 Tg S) and DMS (0.58 Tg S) with the remainder as DMDS (0.13 Tg S), COS (0.12 Tg S) and CS₂ (0.07 Tg S). Our value of 1.7 Tg S for the total global release from marshes is about half the 4.1 Tg S estimated by Aneja *et al.*¹¹ for the

mean average global emission from saline marshes. In comparison, releases from the coastal regions which includes marshes, estuaries and coastal waters given in the most recent global budgets^{12,13} range from 5 to 12 Tg S yr^{-1} . Thus, marshes by our estimate could contribute between 14 and 34% of the coastal estimates. Emissions from estuarine and coastal waters also contribute to the coastal region flux but the sulphur speciation and rates of release would probably be different than the sulphur gases emitted from marsh creek waters with more DMS¹⁴ and much less H₂S released. However, there have not been any detailed emission studies from estuarine or coastal waters to verify this hypothesis. Recent estimates of the total global biotic release^{13,15,16} range from 32 to 103 Tg S yr^{-1} . Marshes, therefore, would contribute only a small portion (1.6–5.4%) to the global biogenic emission.

Table 2 Annual gaseous sulphur emissions from natural sources

Source	Sulphur compound ($\text{g S m}^{-2} \text{ yr}^{-1}$)					Total
	H ₂ S	DMS	COS	CS ₂	DMDS	
Oceanic	—	0.105 ¹⁶	0.001 ^{25,26}	0.00083 ²⁷	—	0.1069
Inland non-swamp soils (derived from ref. 8)	0.0970	0.0070	0.0156	0.0232	0.0016	0.1444
Salt marshes (this paper)	2.19	1.52	0.32	0.18	0.34	4.55

While the emissions of COS and CS₂ are only a minor fraction (9.6%) of the total gaseous sulphur release they may have an important role globally. One reason is because COS and CS₂ are long-lived sulphur species with tropospheric lifetimes of ~1 yr and 1 month, respectively. Carbonyl sulphide, the most abundant sulphur gas in the atmosphere¹⁷, has been proposed by Crutzen¹⁸ to be the dominant sulphur source for the stratospheric aerosol layer during non-volcanic periods. An additional atmospheric source of COS results from the photooxidation of some portion of the CS₂ in the troposphere¹⁹ because photooxidation of CS₂ yields one molecule each of COS and SO₂. Table 3 inventories COS, CS₂ and total sulphur emissions from natural sources including our estimate from marshes. The continental soil emissions were calculated using a global area²⁰ of 120.5×10^{12} m² (excluding lakes, streams, swamps, rocks, ice and sand) and the data in Table 2. We used 367.1×10^{12} m² as the global area of the oceans¹⁶. We estimate that marshes may release a total of $0.16 \text{ Tg S yr}^{-1}$ (COS and CS₂ converted to COS) to the troposphere. The release from marshes is approximately two-fold larger than the quantity needed to maintain the stratospheric sulphate aerosol, but inland soils appear to be an even larger source.

We believe there is a global natural release of $\sim 5.6 \text{ Tg S yr}^{-1}$ as COS plus CS₂ (Table 3) which is in good agreement with several recent estimates of the total annual emission (natural plus anthropogenic) of $\sim 5 \text{ Tg S}$ (refs 12, 21). Thus, natural and, especially, biogenic sources appear to dominate the global atmospheric COS plus CS₂ cycle. Inland soils appear to be the major biotic source of COS plus CS₂ (84%) to the troposphere with about 4.7 Tg S yr^{-1} released from this source (Table 3). Our estimate of 3.2 Tg S yr^{-1} for the biogenic emission of CS₂ falls within the range of 1.5–5.8 Tg S calculated for a total global CS₂ source by Wine *et al.*¹⁹ from atmospheric CS₂ abundances and modelling. If all the CS₂ is converted to COS, this would increase the natural tropospheric COS source to a maximum of $\sim 4.0 \text{ Tg S yr}^{-1}$ available for reaction with OH (ref. 22), long wavelength photodissociation²³ and other sinks or for transport of $\sim 0.08 \text{ Tg S yr}^{-1}$ to the stratosphere. Our estimate of 2.4 Tg S yr^{-1} for the direct COS emission from biogenic sources is much larger than the 0.5 Tg S yr^{-1} estimated by Turco *et al.*²¹ to arise from biospheric processes. The difference in the estimates is due to our inclusion of recent data on the releases from marshes and soils and the change of the oceans from a sink to a source of these gases.

From the data in Table 2, we calculate that there is a global biogenic release of $\sim 58 \text{ Tg S yr}^{-1}$ to the atmosphere (Table 3). This value is well within the three-fold range of 32–103 Tg S yr⁻¹ cited previously. Our global biogenic estimate is about half the recently calculated global anthropogenic SO₂ emission of 113 Tg S yr^{-1} (ref. 12). Biogenic oceanic emissions might be as large as 39 Tg S yr^{-1} predominately as DMS (98%) while inland soils may release $\sim 17 \text{ Tg S yr}^{-1}$ mostly as H₂S (67%). Ryaboshapko¹², in a recent global sulphur budget, calculated an annual bioemission from the land surface of 16 Tg S which is very close to our soils flux value (Table 3). Our estimate of the H₂S flux from soils of $11.7 \text{ Tg S yr}^{-1}$ compares well with the 14 Tg S of H₂S estimated by Delmas *et al.*²⁴ to be emitted from aerobic soils. Our estimates suggest that oceanic DMS fluxes might be the single largest natural source (66%) of biogenic sulphur to the lower troposphere.

Emission of gaseous sulphur compounds from salt marshes are higher, per unit area, by one or two orders of magnitude than emissions from other ecosystems. However, because of the small global area of salt marshes, gaseous sulphur emissions from this source appear to contribute a minor fraction to the global emissions. Saline marsh releases of the short-lived sulphur

Table 3 Global release of COS, CS₂ and total sulphur emissions from natural sources (Tg S yr⁻¹)

Source	COS	CS ₂	Total sulphur emissions
Oceans ^{25–27}	0.40	0.30	39.2
Wild fires in temperate and boreal forests ²⁸	0.01	—	—
Inland non-swamp soils	1.88	2.80	17.4
Salt marshes	0.12	0.07	1.7
Volcanoes and fumaroles ²⁹	0.01	—	—
Total	2.42	3.17	58.3

gases (DMS, H₂S and DMDS) may have an important role in the atmospheric sulphur cycle on a local scale where the areal extent of marshes is large. Biotic emissions seem to be the dominant global source of long-lived sulphur gases, COS and CS₂, with a major fraction of the release originating from inland soils. Additional measurements of sulphur gas emissions from coastal oceans, soils (especially tropical soils) and forests are needed to refine the global biogenic emission estimates.

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Functional neuronal replacement by grafted striatal neurones in the ibotenic acid-lesioned rat striatum

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In rats, striatal neuronal destruction by so-called excitotoxic amino acids, kainic acid or ibotenic acid (IA) produce neuropathological and neurochemical changes in the basal ganglia¹⁻⁴ which resemble those seen in patients with Huntington's chorea^{5,6}. Such lesioned animals show a behavioural syndrome which is reminiscent of the cardinal symptoms of the disease⁷⁻¹², accompanied by a substantial increase in local cerebral metabolic activity in several striatal target structures within the extrapyramidal motor system¹³⁻¹⁵. The study was designed to explore the potential of grafted fetal striatal neurones implanted into the IA-lesioned striatum to compensate for the structural, neurochemical, metabolic and behavioural defects of IA-lesioned rats. Extending previous studies^{16,17}, we report here that such striatal implants can significantly ameliorate the lesion-induced locomotor hyperactivity and at least partly normalize the metabolic hyperactivity in the extrapyramidal neuronal system.

Our experimental rats (Fig. 1) was divided into three groups. One group, consisting of five lesioned, five lesioned and grafted and seven unoperated control rats, was used for measurements of local glucose utilization, according to the autoradiographic ¹⁴C-2-D-deoxyglucose (¹⁴C-2-DG) method of Sokoloff *et al.*¹⁸. We also used these brains for histology and morphometry of the grafts and the host striatal structures. The second group, consisting of six lesioned and five lesioned and grafted rats, was used for measurements of glutamic acid decarboxylase (GAD)¹⁹ in the graft and the host striatum on the lesioned and control sides. The third group, consisting of nine lesioned, ten lesioned and grafted and six control rats, was used for analysis of locomotor activity.

The IA lesion caused an average reduction of ~65% in the volume of the head of the caudate-putamen accompanied by an average sevenfold increase in the volume of the lateral ventricle at the same cross-sectional levels (Fig. 1). The Klüver-Barrera stain revealed a severe loss of striatal neurones; only about 5-10% of the original volume of the head of the caudate-putamen showed sparing of neurones. The remaining tissue was virtually free of stainable neurones and had the appearance of a glial scar penetrated by the seemingly undamaged fibre bundles of the internal capsule. The spared neurones occurred as a rim along the ventricular surface and along the external capsule in the caudal aspects of the lesion (Fig. 1*b*). The nucleus accumbens and the tail of the caudate-putamen were only marginally affected; in most cases there were only minor signs of cell damage along the needle tracks in the cortex. Probably as a secondary consequence of the lesion^{6,20}, both globus pallidus and pars reticulata of the substantia nigra underwent a marked shrinkage on the lesioned side^{20,21}.

The GAD assay revealed an average depletion of the γ -aminobutyric acid (GABA) synthesizing enzyme concentration of 58% in the head of the caudate-putamen encompassing the lesion, from 76.9 ± 5.5 to 32.3 ± 8.4 nmol per h per mg protein. Taking into account the volume reduction of the caudate-putamen, we estimated the depletion of the total GAD content in the head of the caudate-putamen to be ~85%.

The striatal implant had an average volume of about 4 mm³ (range 2-7 mm³), which resulted in an average increase of 50% in the lesioned caudate-putamen volume. The amount of caudate tissue with signs of sparing, and the volume of the scar tissue

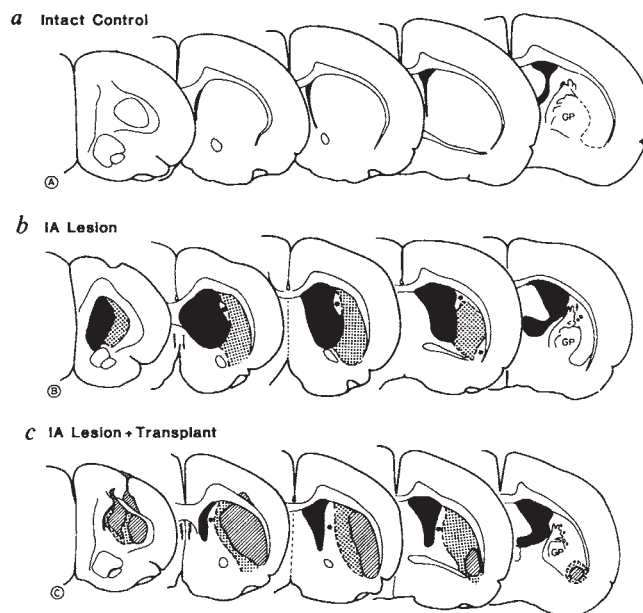


Fig. 1 Camera lucida drawings of a normal control rat (*a*), an IA-lesioned rat (*b*) and an IA-lesioned and grafted rat (*c*) 11 weeks after the lesion. The double-cross-hatched areas represent the scar tissue, the asterisks the areas of neuronal sparing and the black areas the lateral ventricle. The graft tissue in *c* is represented by the single-cross-hatched area. GP, globus pallidus.

Methods: 53 Female Sprague-Dawley rats, 180-200 g in weight, were used in this study. 13 Rats were unoperated controls; the remaining 40 were anaesthetized with methohexital barbiturate (Lilly) and mounted in a stereotaxic frame. Part of the cranium anterolateral to bregma was carefully removed by drilling, and a total of 20 μ g of IA (Natural Products Ltd) in 1 μ l of phosphate buffer (pH 7.4) was injected at four stereotaxically defined sites in the right caudate-putamen (A 0.4, L 3.9, V 5.3; A 0.4, L 3.1, V 4.0; A 1.3, L 3.1, V 4.4; A 1.3, L 2.0, V 4.3; all coordinates in mm, anterior and lateral coordinates from bregma, ventral coordinates from dural surface). Each of the four 0.25- μ l microinjections was performed over a total of 10 min before the needle was slowly withdrawn. 5-7 Days after the IA lesion, 20 of the rats were selected at random to receive implantations of cell suspensions prepared from primordial striatum taken from rat fetuses of 13-15 days gestational age, as described previously¹⁶. Primordial striatal tissue was collected in 0.6% glucose in saline at room temperature and then transferred to a similar glucose solution with the addition of 0.1% trypsin (crude type II, Sigma), in which incubation took place at 37 °C for 15-20 min. At the end of the incubation period the trypsin medium was removed and the tissue rinsed four times with fresh glucose-saline solution. The tissue pieces were then mechanically dissociated with a fire-polished Pasteur pipette (diameter ~1 mm) in a volume equivalent to 10 μ l per two striatal primordia. 1 μ l suspension was injected over 1 min at each of the four sites which had previously received IA. After each injection the needle was left in position for an additional 2 min before being slowly withdrawn.

were very similar in the lesion and the lesion + graft groups (5% compared with 6%; and 28% compared with 31%, respectively; Fig. 1*b, c*). Microscopically, the implant appeared as one or several neurone-rich cell masses sometimes extending along the needle tracks up into the overlying cortex (Fig. 1*c*). In the animals taken for neurochemical analysis, we found that the GAD level of the graft tissue was as high as that of the normal caudate-putamen (83.4 ± 9.0 nmol per h per mg protein), and in a parallel immunohistochemical study²¹ the striatal grafts contained many of the chemically identified neurone types normally present in the neostriatum.

In the ¹⁴C-2-DG autoradiography (Table 1), we found that the IA lesion caused a 60% reduction in glucose utilization throughout the lesioned caudate-putamen. The partially spared medial portion displayed smaller reductions (22%), while the

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Table 1 Transplant-induced effects on regional ^{14}C -2-deoxy-D-glucose utilization in the awake IA-lesioned rat

Structures	Intact	Ibotenate lesion		Ibotenate lesion + striatal transplant	
		Ipsilateral	Contralateral	Ipsilateral	Contralateral
Host caudate-putamen					
Medial	68 ± 1	53 ± 6*	87 ± 2*	49 ± 4*	75 ± 2†
Lateral	68 ± 1	28 ± 2*	87 ± 2*	30 ± 3*	74 ± 1*†
Tail	68 ± 1	69 ± 8	81 ± 4*	64 ± 2	71 ± 1†
Primary striatal targets					
Globus pallidus	42 ± 2	53 ± 1*	55 ± 3*	45 ± 2†	51 ± 2*
Entopeduncular nucleus	42 ± 2	56 ± 3*	47 ± 4	49 ± 3*	43 ± 1
Substantia nigra, reticulata	41 ± 1	61 ± 3*	53 ± 3*	58 ± 2*	51 ± 3*
Substantia nigra, compacta	52 ± 1	59 ± 2*	59 ± 3*	56 ± 1*	57 ± 2*
Secondary striatal targets					
Subthalamic nucleus	63 ± 1	74 ± 2*	75 ± 3*	66 ± 3†	71 ± 2*
Lateral habenula	89 ± 2	103 ± 7*	100 ± 5*	106 ± 4*	105 ± 1*
Ventrolateral thalamus	64 ± 1	75 ± 4*	79 ± 4*	71 ± 2*	74 ± 2*
Ventromedial thalamus	79 ± 2	79 ± 3	91 ± 2*	76 ± 2	86 ± 2*
Superior colliculus, deep	64 ± 1	75 ± 1*	76 ± 2*	68 ± 1†	71 ± 2*†
Associated motor and limbic areas					
Nucleus accumbens	66 ± 2	76 ± 2*	81 ± 2*	67 ± 3†	73 ± 3*†
Ventral tegmental area	39 ± 1	50 ± 2*	49 ± 2*	43 ± 2†	44 ± 2
Nucleus ruber	54 ± 3	67 ± 2*	69 ± 2*	65 ± 2*	65 ± 3*
Mesencephalic raphe	73 ± 1	—	71 ± 4	—	77 ± 2
Sensorimotor cortex	79 ± 1	89 ± 7*	97 ± 4*	83 ± 3	91 ± 1*
Anterior cingulate cortex	79 ± 2	87 ± 5	93 ± 6	77 ± 4	86 ± 4
Pontine nuclei	52 ± 1	56 ± 3	55 ± 3	54 ± 1	54 ± 1
Cerebellar hemisphere	51 ± 3	56 ± 2	57 ± 2	52 ± 2	52 ± 2
Cerebellar nuclei	67 ± 4	79 ± 3*	79 ± 3*	74 ± 2	74 ± 2
White matter	31 ± 2	34 ± 2	31 ± 2	32 ± 2	32 ± 2

Local ^{14}C -2-deoxy-D-glucose utilization after unilateral IA lesion ($n = 5$) or IA lesion plus striatal suspension grafts ($n = 5$). A group of seven age- and weight-matched unoperated female rats served as the control group. No side-to-side differences were observed within any brain structure in the intact animals, so the two sides were pooled to provide a single control value. Structures measured were sampled according to Schwartz and Sharp³¹. No transplant-induced effects were observed in other brain areas.

Methods: Under light halothane anaesthesia (1% in a 7:3 $\text{N}_2\text{O}_2:\text{O}_2$ gas mixture), both femoral arteries and one femoral vein were cannulated. Groin incision sites were infiltrated with local anaesthetic (xylocaine) and sutured. A loose-fitting plaster cast was applied around the lower body and hind legs, taking care not to impair thoracic movements, and the cast was taped to lead weights. Thus restrained, anaesthesia was discontinued and at least 2 h were allowed to elapse before proceeding with the experiments. The measurement was initiated with a 30-s intravenous injection of 0.5 ml ^{14}C -labelled 2-deoxy-D-glucose ($125 \mu\text{Ci kg}^{-1}$, NEN). Over the subsequent 45 min, a total of 14 timed arterial blood samples were collected and the plasma separated from the cellular fraction by immediate centrifugation. Aliquots of the plasma were taken for measurement of glucose levels ($10 \mu\text{l}$) and ^{14}C concentrations ($20 \mu\text{l}$). At the end of 45 min the animals were decapitated and their brains were rapidly dissected and frozen in isopentane at -50°C . Autoradiograms were prepared from ~150 coronal cryostat sections ($20 \mu\text{m}$) from each brain mounted on glass coverslips. The sections were placed in a light-tight X-ray cassette, together with a set of seven ^{14}C -containing plastic standards (concentrations 48–1,061 nCi per g tissue equivalents) in close apposition to a sheet of X-ray film (Kodak SB-5) for 7 days. The processed films were analysed densitometrically using a Leitz TAS plus computerized densitometer. Local cerebral glucose utilization was calculated according to Sokoloff *et al.*¹⁸.

* Significantly different from intact control value ($P < 0.05$); †, significantly different from IA lesion alone ($P < 0.05$). One-way analysis of variance with post-hoc Neuman–Keuls test. No symbol indicates $P > 0.05$.

spared tail of the caudate was unaffected. In contrast to the ipsilateral decreases, the contralateral caudate-putamen displayed increased mean glucose utilization values of between 19 and 28% over intact control levels in the different caudate regions measured. All major primary and secondary projection areas of the neostriatal efferents (Fig. 2) showed significant uni- or bilateral increases in glucose use. The strongest increases were seen in the pars reticulata of the substantia nigra (+49% and 29% on the ipsi- and contralateral sides, respectively), in the globus pallidus (+26% and 29%), in the subthalamic nucleus (+18% and 19%) and in the deep superior colliculus (+17% and 18%). Bilateral increases were seen also in several areas functionally associated with the extrapyramidal motor system, such as the ventral tegmental area, nucleus accumbens, sensorimotor cortex and nucleus ruber, whereas other cortical and brain-stem regions remained unaffected.

The striatal implant proper exhibited a significant ^{14}C -2-DG utilization of $69 \pm 3\%$ of the intact control neostriatum. The implant does not influence glucose use in the surrounding, lesioned or spared areas of the host caudate-putamen, although the contralateral increases in glucose use in animals with IA lesions alone were attenuated enough to be no longer significantly different from the intact controls (Table 1).

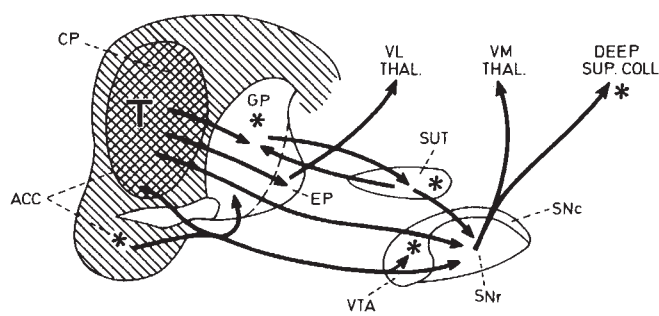


Fig. 2 Principal arrangements of the major output pathways from the caudate-putamen and nucleus accumbens. Those primary and secondary targets which exhibited graft-induced reductions in ^{14}C -2-DG utilization are marked by asterisks. ACC, nucleus accumbens; CP, caudate-putamen; EP, entopeduncular nucleus; GP, globus pallidus; SN, substantia nigra (c, compacta, r, reticulata); SUT, subthalamic nucleus; VL and VM Thal, ventrolateral and ventromedial thalamic nuclei; VTA, ventral tegmental area.

The hypermetabolic responses in the primary and secondary striatal projection areas were normalized by the implant ipsilaterally in the globus pallidus and subthalamic nucleus and bilaterally in the deep superior colliculi ($\dagger$ in Table 1; * in Fig. 2). In the functionally associated areas, a significant transplant-induced effect was also seen in the VTA and nucleus accumbens. No transplant-induced effects were observed elsewhere in the brain.

Locomotor activity was measured in two different tests (Table 2). Previous studies^{8,10} have shown that rats with excitotoxic striatal lesions are hyperactive only when tested during their active period at night (when rats normally express about 90% of their diurnal locomotor activity²²) or under food deprivation. Consistent with these reports, the IA-lesioned animals in the present study were significantly hyperactive in the open field when tested overnight under temporary (24 h) food and water deprivation. In agreement with previous findings¹⁷, the locomotor hyperactivity was compensated for completely in the animals with IA lesions plus striatal transplants ($P < 0.01$). These effects were also confirmed when locomotor activity was measured in rotometer bowls (Table 2); the individual scores recorded in the two tests were highly correlated ($r = 0.8$; $t = 5.05$). The rotometer test showed, moreover, that although the unilateral IA lesion caused spontaneous turning behaviour (in the direction towards the lesion) during the first few days after lesion, the motor asymmetry was no longer evident in any of the lesioned or grafted groups by 10–12 weeks after the operation.

The present results thus demonstrate that the striatal suspension grafts not only survive and differentiate within the host neostriatum previously depleted of its constituent neurones by IA, but that they are also capable of compensating for the nocturnal locomotor hyperactivity and the extrapyramidal metabolic hyperactivity seen in the unilaterally IA-lesioned animals. The exact mechanism of these transplant-induced effects are unknown. Previous experiments with implants of catecholamine-producing cell systems^{23–28} have provided evidence that intracerebral grafts can exert their functional effects in the host brain both via direct axonal connections with previously denervated elements in the host target, and via a more diffuse release of the active compound into the host tissue or the cerebrospinal fluid. Both these mechanisms seem consistent with our present results. It thus seems possible that the striatal implants could exert a neurohumoral-like effect either on spared neurones in the caudate-putamen, or on parts of the nucleus accumbens in the neighbourhood of the graft, or perhaps even on more distant sites via a diffuse release of active compounds into the cerebrospinal fluid in the adjoining lateral ventricle. Alternatively, the grafted neurones might function via fibre connections established with striatal neurones in the host, either, for example, via a local fibre outgrowth into the spared portions of the striatum or nucleus accumbens, or via fibres growing over somewhat longer distances to reach, for example, the globus pallidus. Evidence for such effects of the striatal implants will, however, require further studies on the connectivity of the grafted neurones.

Table 2 Transplant-induced effects on locomotor activity of IA-lesioned rats

Groups	Overnight activity (total photocell counts)	Overnight turning in rotometer	
		Total half-turns	Side-bias (% turns in ipsilateral direction)
Control	7,232 $\pm$ 380	142.0 $\pm$ 28.7	44.8 $\pm$ 9.6
IA lesion	12,273 $\pm$ 1,842*	336.0 $\pm$ 49.4*	58.7 $\pm$ 8.2
IA lesion + transplant	6,485 $\pm$ 467†	170.0 $\pm$ 27.2†	49.8 $\pm$ 8.2

Overnight locomotor activity measured in enclosed automated activity boxes (50 $\times$ 50 cm), connected to a microprocessor. Values represent the mean total photocell counts $\pm$ s.e.m. over a 13-h test period (1830–0730 h). 10–11 weeks after IA lesion and transplantation, rats from the three groups were randomly selected for activity tests. Before the test session, rats were temporarily deprived of food and water for 24 h (see text). Statistical analysis: one-way analysis of variance, $F = 7.25$, post-hoc Neuman-Keuls. Overnight locomotor activity was measured in rotometer bowls of diameter 55 cm. Values represent the total number of half-turns $\pm$ s.e.m. over an 11-h period (1830–0530 h). Rats were temporarily deprived of food and water for 2–5 h before testing. Statistical analysis, one-way analysis of variance, $F = 6.33$, post-hoc Neuman-Keuls. Side-bias is expressed as turns in the ipsilateral direction to IA lesions divided by the total number of turns. Values show mean percent $\pm$ s.e.m. for each group. One-way analysis of variance, $F = 0.59$.

* $P < 0.05$ compared with control; † $P < 0.05$ compared with IA lesion.

The increased glucose utilization seen in several primary and secondary neostriatal target areas in the IA-lesioned animals indicates that the neostriatum normally exerts an inhibitory tonic action on the functional activity of these structures. Interestingly, it has previously been shown that the increases in glucose utilization induced by excitotoxic lesions of the neostriatum can be attenuated by systemic injections of the GABA receptor agonist, muscimol²⁹, and that it is possible to modify locomotor activity in rats by local administration of GABA receptor-active drugs directly into the globus pallidus³⁰. Against this background it may be sufficient for the striatal implants to reinstate a tonic and relatively nonspecific inhibition over the striatal output structures (perhaps via release of GABA or other neuromodulators from the graft) in order to compensate for the lesion-induced metabolic and locomotor hyperactivity in the IA-lesioned animals. More complex striatum-dependent behaviours, may, by contrast, require both temporally and spatially patterned cortical inputs and are thus less likely to be influenced by the implants unless they are capable of establishing ordered and relatively specific afferent and efferent connections with the host brain. Whether this will be possible to achieve with striatal implants is an interesting topic for further investigation.

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The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems

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The striatum (caudate-putamen) of the basal ganglia in the mammalian forebrain is a mosaic of two interdigitating, neurochemically distinct compartments. One type, the 'patch' compartment, is identified by patches of dense opiate receptor binding^{1,2}, and is enriched in enkephalin- and substance P-like immunoreactivity³. The other compartment, the 'matrix', has a high acetylcholinesterase activity^{2,4}, and is shown here to have a dense plexus of fibres displaying somatostatin-like immunoreactivity. The present study demonstrates the two compartments have distinct connections, using a method that concurrently reveals striatal input, output and neurochemical systems in the rat. Patches receive inputs from the prelimbic cortex (a medial frontal cortical area with direct 'limbic' inputs from the amygdala⁵ and hippocampus^{6,7}); they also project to the substantia nigra pars compacta (the source of the nigrostriatal dopaminergic system). Conversely, the matrix receives inputs from sensory and motor cortical areas⁸; here it is shown to project to the substantia nigra pars reticulata (the source of the non-dopaminergic nigrothalamic and nigroreticular system^{9,10}). Also, an intrinsic striatal somatostatin-immunoreactive system is described that may provide a link between the two compartments. The striatal patch and matrix compartments thus appear to be functionally distinct and interactive parallel input-output processing channels.

The input-output organization of the striatum was examined using a method that concurrently demonstrates corticostriate

systems (*Phaseolus vulgaris* leucoagglutinin (PHA-L) anterograde tracing¹¹), striatonigral projection neurones (fluorescent retrograde tracer Fast blue¹²), and either the striatal patch (with enkephalin- or substance P-immunoreactivity³) or matrix (with somatostatin-immunoreactivity¹³) compartments. The anterogradely transported plant lectin PHA-L was injected iontophoretically into the right prelimbic cortex of eight female albino rats, as previously described¹¹. These animals were also injected with 50 nl of the retrogradely transported fluorescent tracer Fast blue (in a 2.5% suspension) into the right substantia nigra. The animals received a left intraventricular injection of 75–100 µg colchicine 8 days after the tracer injections and 2 days later were perfused under chloral hydrate anaesthesia with 0.9% saline followed by 4% formaldehyde in 0.1 M acetate buffer (pH 6.5) and then 4% formaldehyde in 0.1 M borate buffer (pH 9.5) (ref. 14). The brains were removed and stored overnight in the last solution plus 20% sucrose. Coronal 30-µm-thick sections were cut on a freezing microtome, and sections through the substantia nigra examined to reconstruct the site of the Fast blue injections. The other sections were incubated overnight at 4°C in 0.4% Triton X-100 plus 2% normal goat serum (NGS) in 0.02 M potassium phosphate-buffered saline (KPBS, pH 7.4), and then incubated at 4°C for 24–48 h in a 1:1,000 dilution of guinea pig antisera directed against PHA-L in KPBS plus 2% NGS. These sections were then rinsed in KPBS and incubated at 4°C for 24–48 h in rabbit antisera directed against either substance-P (1:3,000, a gift of M. Brown), Leu-enkephalin (1:2,000, Immunonuclear, Inc.), or somatostatin¹³ (SS28 (1–12), 1:5,000 + SS28, 1:2,000, a gift of R. Benoit). After rinsing for 30 min, the primary antisera were localized by incubating the sections in a mixture of affinity purified fluorescein-labelled goat anti-guinea pig IgG (GAGP-FITC, 1:100, Cappel) and affinity purified tetramethylrhodamine-labelled goat anti-rabbit IgG (GAR-TRITC, 1:100; Sigma) in KPBS plus 0.3% Triton X-100 and 2% NGS for 45 min. Sections were examined with a Nikon fluorescent microscope. Sections incubated with one or the other labelled antisera demonstrated no cross-reactivity with the inappropriate primary antisera. Pre-adsorption of each antiserum with 1 mg ml⁻¹ of the homologous

* Present address.

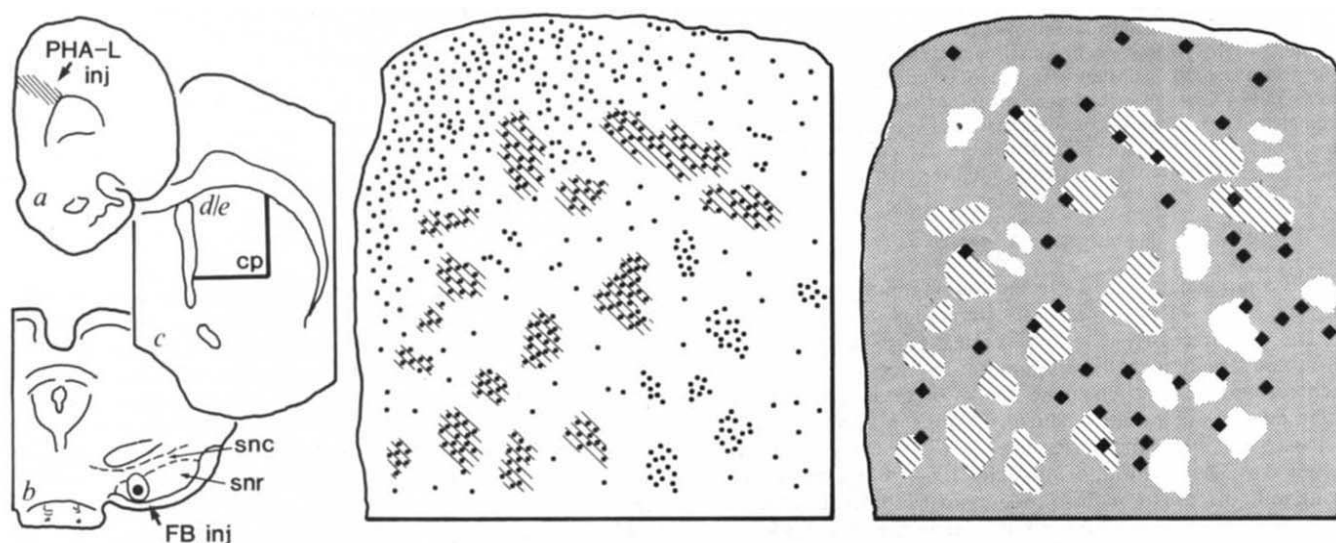


Fig. 1 Diagrammatic representation of labelling in coronal sections from case CSI-4. *a*, The location of PHA-L injection site (PHA-L inj) in the prelimbic cortex shown by hatching. *b*, The location of the Fast blue injection (FB inj) centred in the ventro-medial substantia nigra pars reticulata (snr) with spread into the pars compacta is marked (○). The area within the medio-dorsal caudate-putamen (cp) outlined in section *c* is enlarged in *d* and *e*. *d*, Retrogradely labelled striatonigral projection neurones (dots) are most heavily concentrated in the medio-dorsal area. Their distribution here is relatively homogeneous, but ventral distribution is in discrete clusters, some of which are co-extensive with patches of labelled corticostriate afferents marked with hatching. *e*, The concurrent localization of somatostatin-immunoreactive cell bodies (diamonds) shows that such neurones are located both within and around patches of PHA-L labelled corticostriate input (hatching), but that somatostatin-immunoreactive fibres (shaded area) appear to be limited to the matrix zone complementary to such patches. In this example, the prelimbic input is primarily to the medial half of the striatum; in two other cases where the PHA-L injection was into a more rostral part of the prelimbic cortex, and labelled corticostriate inputs are distributed to the patches in the lateral half of the striatum.

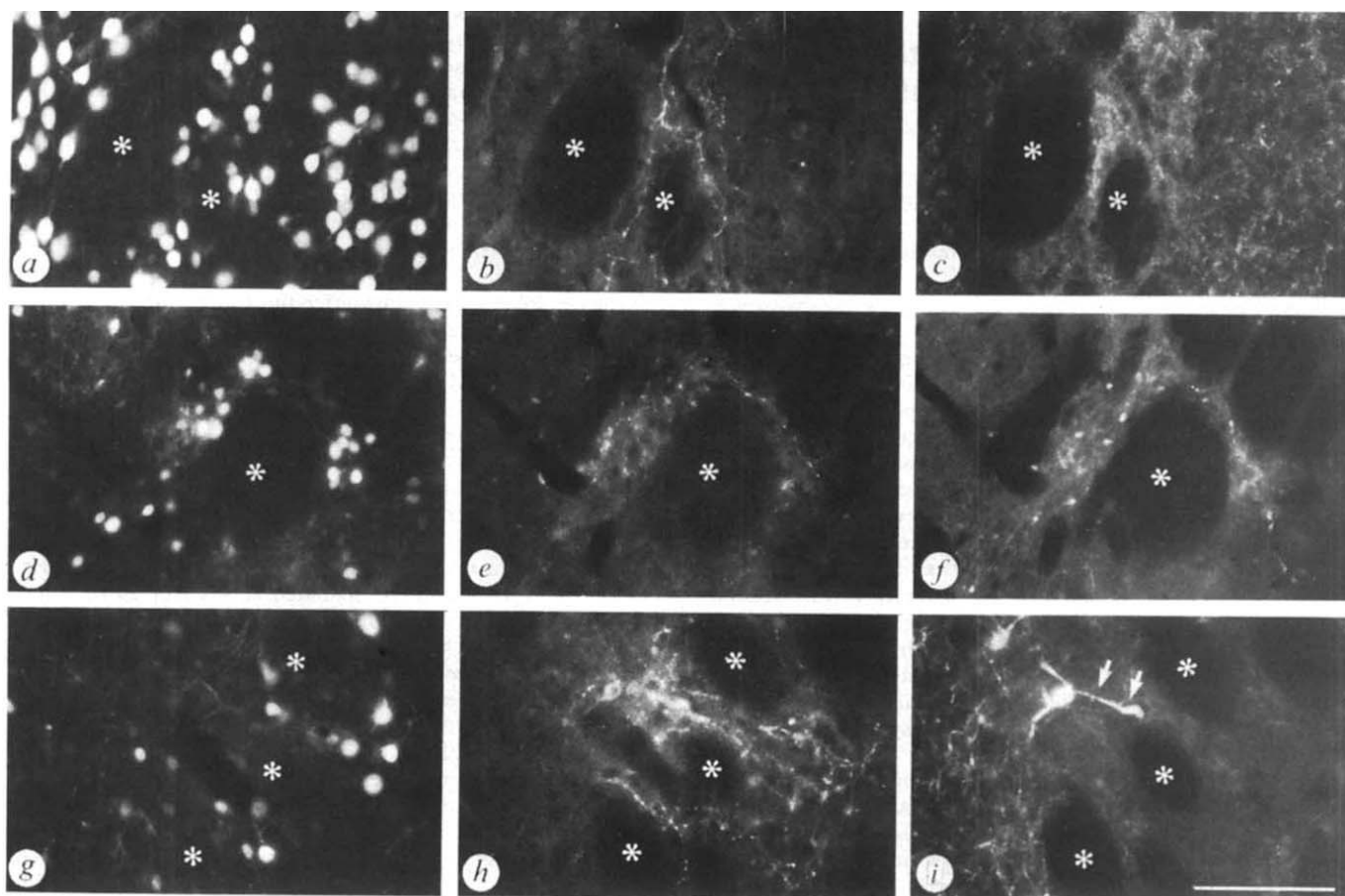


Fig. 2 Photomicrographs of striatal areas in three sections, *a-c*, *d-f*, and *g-i*, from case CSI-4 showing concurrent localization of striatonigral projection neurones labelled with the fluorescent marker Fast blue (*a*, *d*, *g*); patches of corticostriate input labelled with PHA-L (*b*, *e*, *h*) demonstrated with fluorescein-labelled goat anti-guinea pig IgG (GAGP-FITC) and Leu-enkephalin (*c*), substance-P (*f*), and somatostatin-like (*i*) immunoreactivity demonstrated with tetramethylrhodamine-labelled goat anti-rabbit IgG (GAR-TRITC). In each section the three labels are separately revealed with the appropriate epifluorescent excitation filters (e.f.) and barrier filters (b.f.) of the following wavelengths: true blue (e.f., 330–380; b.f., 420), FITC (e.f., 460–485; b.f., 515–545) and TRITC (e.f., 535–550; b.f., 580). Asterisks mark unlabelled cortico-fugal fibre tracts used as landmarks to compare the relative distribution of the labelled structures in the same field. The striatal area *a-c* lies in the medio-dorsal striatum. Fast blue labelled neurones (*a*) are present both within the corticoreceptive (*b*)/Leu-enkephalin-immunoreactive rich (*c*) patch and in the surrounding matrix compartment. The area in another section (*d-f*) is in a more ventral part of the striatum, where a cluster of retrogradely labelled striatonigral neurones (*d*) is co-extensive with a patch of PHA-L labelled corticostriate fibres and terminals (*e*) and a patch of substance P-immunoreactivity (*f*). In an adjacent section (*g-i*), in the same area of the striatum as *d-f*, a cluster of retrogradely labelled striatonigral neurones is observed (*g*), co-extensive with labelled corticostriate afferents (*h*) which are distributed complementary to the surrounding matrix of somatostatin-like immunoreactive fibres and terminals (*i*). A single somatostatin immunoreactive neurone is observed at the border of the corticoreceptive patch area with a dendrite (arrows) the end of which is enlarged due to focal aberration) that extends into the patch area away from the somatostatin fibre plexus in the matrix. Scale bar, 100 μ m.

peptide completely blocked specific staining in the neostriatum.

In six cases, PHA-L injections were localized to different 300- μ m-wide areas in the prelimbic cortex⁵ (Fig. 1*a*). Projections distributed throughout the striatum but restricted to the striatal patch system are seen when the results of these injections are combined. In the example shown in Figs 1 and 2, labelled corticostriate fibres and terminals are distributed in discrete patches, 5–15 per section, of diameter 100–400 μ m and spread throughout the dorso-ventral and rostro-caudal extent of the medial half of the striatum. In this example, the Fast blue injection is centred in the medial substantia nigra pars reticulata with tracer spreading dorsally into the pars compacta (Fig. 1*b*). In the medio-dorsal striatum, retrogradely labelled neurones are homogeneously distributed in both the patch areas co-extensive with labelled prelimbic corticostriate inputs and in the complementary matrix (Figs 1*d*, 2*a-c*). Ventral to this area, labelled striatonigral projection neurones are distributed in discrete clusters (Fig. 2*d*, *g*) co-extensive with the patches of labelled corticostriate terminals (Fig. 2*e*, *h*). These patches (Fig. 2*b*, *e*) are always co-extensive with patches of strong Leu-enkephalin-

immunoreactivity (Fig. 2*c*) and substance P-immunoreactivity (Fig. 2*f*). In a third series of sections, a somatostatin-immunoreactive fibre plexus appears only in the matrix compartment (Figs 1*e*, 2*g-i*). Somatostatin-immunoreactive neurones are present in both patches and matrix and often dendrites from matrix-labelled neurones extend into the patches. Labelled axons of patch somatostatin-immunoreactive neurones project into the surrounding matrix, where they ramify into a fibre plexus which has numerous 'terminal-like' swellings. Somatostatin immunoreactivity was never observed in Fast blue labelled striatal projection neurones.

To obtain a more detailed view of the distribution of retrogradely labelled striatonigral projection neurones relative to the striatal compartments, 12 animals were injected with 35 nl of Fast blue into the substantia nigra. Colchicine was administered intraventricularly in a dose of 75 μ g on the sixth post-injection day and the animals were killed on the eighth day, when the brains were processed as described above. Cases were classified as having injections restricted primarily to the pars compacta ($n=3$), pars reticulata ($n=3$), or with equal spread into both



Fig. 3 *a*, A frontal section through the anterior striatum from case SED-4 onto which are mapped the distribution of substance P-like immunoreactive rich patches (hatching) and striatonigral projection neurones (dots), that are retrogradely labelled following an injection of Fast blue into the substantia nigra pars compacta (snc), shown diagrammatically in *b*. snr, Substantia nigra pars reticulata. Note that in the ventral striatum, labelled neurones are distributed in both the striatal patch and matrix compartments (see text), whereas dorsal to this area, labelled neurones are distributed in clusters that are co-extensive with patches of strong substance P-immunoreactivity.

areas ($n = 6$). Figure 3 shows the distribution of retrogradely labelled neurones relative to patches of substance P-immunoreactivity in one experiment in which the injection was centred in the medial pars compacta (snc, Fig. 3*b*). Labelled neurones are most densely distributed in the ventral striatum in both the patch and matrix compartments; outside this ventral zone and extending to the dorsal border of the striatum, labelled neurones are distributed preferentially in clusters co-extensive with patches of substance P-immunoreactivity. This pattern was evident throughout the rostrocaudal extent of the striatum and was typical in cases where injections were centred in the pars compacta. By contrast, when injections were centred in the pars reticulata, labelled neurones are most densely distributed in the matrix compartment throughout the dorsal striatum. However, the effective injection site cannot be determined using retrograde tracers, so these results must be viewed cautiously. In a recent study¹⁵ (and C.R.G., in preparation) using PHA-L anterograde tract tracing, the dorsal striatum has been shown to give rise to a split nigral projection, one to the pars compacta and a separate, denser one to the ventral pars reticulata. This result, combined with the retrograde data presented here, suggests that the projection neurones in the dorsal striatal patches are the source of fibres that innervate the pars compacta. These conclusions are consistent with ultrastructural data which demonstrate convergence of inputs from both the dorsal and ventral striatum onto pars compacta neurones¹⁶.

Classical studies have linked the basal ganglia to sensorimotor function via the movement disorders produced by Parkinson's disease and Huntington's chorea¹⁷. More recently, involvement of this neural system in affective disorders such as schizophrenia

has been suggested¹⁸. These dual functions of the striatum are thought to be regionally segregated to a ventral or 'limbic' striatal region that mediates striatal involvement in affective behaviour, and a dorsal or 'nonlimbic' striatal region involved in sensorimotor function^{19,20}. The topographic organization of certain striatal input-output systems supports this view. The dorsal striatum, composed of the dorso-lateral part of the caudate-putamen, receives neocortical inputs from sensorimotor areas^{21,22} and projects to the dorsal pallidum and substantia nigra pars reticulata^{23,24}; the latter nucleus provides inputs to the thalamus, tectum and midbrain tegmentum^{9,10}. The ventral striatum¹⁹⁻²¹, composed of the nucleus accumbens, adjacent ventral caudate-putamen, and olfactory tubercle, receives limbic inputs from the amygdala and allocortex^{5,6,19,21} and projects to the ventral extension of the pallidum (the substantia innominata, lateral preoptic area and lateral hypothalamus)^{20,25}. Interactions between these striatal subsystems occurs via the convergence of their transynaptic output pathways to the thalamus^{9,26} and the midbrain tegmentum^{9,25,27}, or via ventral striatal input to the substantia nigra pars compacta^{23-25,28}, which provides the nigrostriatal dopaminergic 'feedback' system to the dorsal striatum.

Recently the boundaries of the limbic striatum have been expanded to include the most medial part of the dorsal striatum, which receives inputs from the amygdala²¹ and cingulate cortex²⁹. However, these findings do not address the issue of possible interaction of limbic and non-limbic inputs within any given striatal district. The present results show that the prelimbic cortex, which receives direct limbic inputs from the amygdala⁵ and hippocampus^{6,7}, projects to the patches throughout the striatum including the dorsolateral part that receives sensorimotor cortical inputs^{8,21}. In this striatal region, sensory and motor cortical areas preferentially project to the matrix⁸. This suggests that, on the basis of extrinsic connections, the striatal patch and matrix are functionally distinct compartments. Furthermore, it is shown here that the striatal projection neurones in the patches project to the substantia nigra pars compacta whereas those in the matrix project to the substantia nigra pars reticulata. The somatostatin-immunoreactive intrinsic system provides a possible link between the two striatal compartments, with neurones in the patches providing inputs to the surrounding matrix areas. These findings suggest that the striatal patch and matrix compartments comprise a system of intermixed and interactive functionally distinct parallel input-output processing channels that are overlaid on the more classically recognized topographically ordered dorsal (non-limbic) and ventral (limbic) segregation of striatal connectivity and function^{19-21,30}.

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Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism

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The compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces an irreversible neurological syndrome in man^{1,2} and monkey³ which is similar to idiopathic Parkinson's disease in its clinical, pathological, neurochemical and pharmacological response properties. MPTP is selectively neurotoxic to the dopaminergic regions of the brain, destroying neurones in the substantia nigra (A8 and A9 cells, nigrostriatal system) but not the ventral tegmental area (A10 cells, mesolimbic system)³. Selective dopamine depletion and nigral cell loss after MPTP treatment has also been reported recently in the mouse⁴. The mechanism by which a peripherally administered, low-molecular weight compound exerts permanent but selective toxic effects on dopamine systems in the brain may be relevant to parkinsonian syndromes induced by other toxins and to the disease process in idiopathic Parkinson's disease. We report here that MPTP is oxidized in the brain to a pyridinium species (a compound with potent herbicidal activity) and, in the monkey, is trapped intraneuronally. Furthermore, we demonstrate that this enzymatic oxidation is blocked *in vivo* in the mouse by a monoamine oxidase inhibitor, a condition which also blocks the neurotoxicity⁵, indicating that the oxidative metabolism of MPTP is required for its neurotoxic effect.

Radiolabelled [¹⁻¹⁴C]MPTP was prepared by reacting ¹⁴C-methyl iodide (2 mCi, 5.4 mg) with 4-phenylpyridine (10 mg) in dichloromethane at room temperature for 24 h, followed by reduction of the dried residue in aqueous solution with excess NaBH₄, using the method of Lyle⁶. The product, [¹⁻¹⁴C]MPTP, was extracted into hexane, dried and reconstituted in ethanol for injection. Alternatively, excess 1-methyl-4-phenylpyridinium iodide (5.4 mg) was reduced with NaB³H₄ (77.8 Ci mmol⁻¹, 0.24 mg) in aqueous base at room temperature for 2 h and the product, 2,6-³H₂-MPTP (38 Ci mmol⁻¹), extracted into hexane, dried and reconstituted in ethanol.

Four rhesus monkeys were injected intravenously with various combinations of ³H₂-MPTP, ¹⁴C-MPTP and unlabelled MPTP and killed between 1 and 20 days after injection (Table 1). After a lethal dose of pentobarbital, the brains were removed and bisected in the sagittal plane. The right half was rapidly frozen and processed for autoradiography in the coronal plane. The left half was dissected using gross anatomical landmarks and the pieces frozen and retained for chemical studies. Dissected regions were homogenized in water (1:4 w/v) and either solubilized with Protosol (NEN) for counting or extracted with ethanol for counting and HPLC of radioactive materials, as described in Fig. 1 legend. Twenty-eight adult male mice (NIH Swiss Webster, 20–22 g) were also used. Seven mice were injected intraperitoneally (i.p.) with saline and seven with pargyline

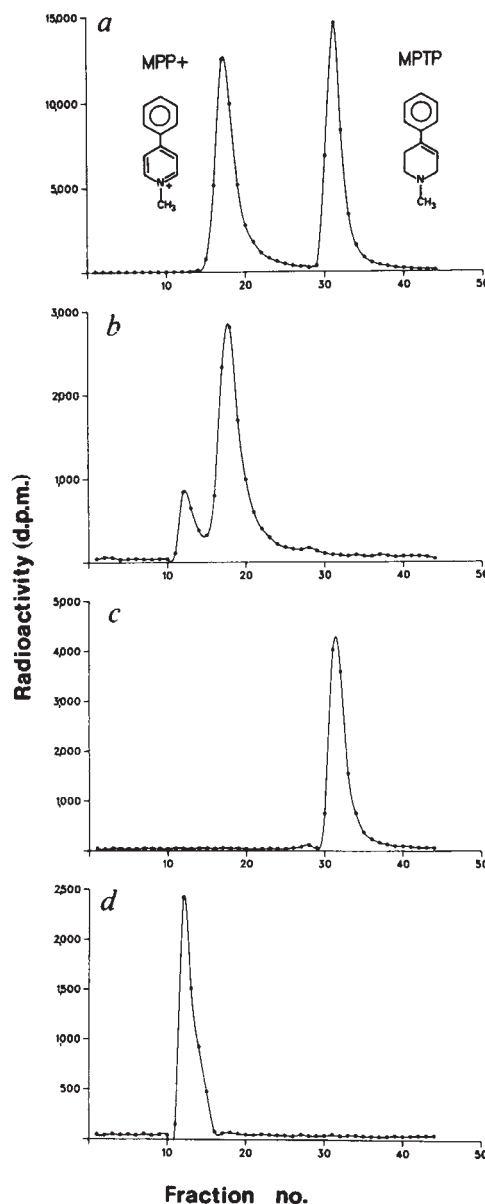


Fig. 1 *a*, Elution profiles of ³H-MPTP and ¹⁴C-MPP⁺. *b*, Elution profile of radioactivity extracted from the brain of monkey BZ9. The predominant ³H peak extracted from the monkey brain co-eluted with MPP⁺ (fractions 16–24). The elution profile in *c* is that of hexane-extractable ³H-labelled material obtained after NaBH₄ reduction of 50 µl of an ethanol extract. This reduced compound eluted as a single peak coincident with MPTP. The radio-chromatogram in *d* is for cerebrospinal fluid from monkey BZ9 taken at the time of killing. The absence in CSF of MPP⁺, which predominates in brain, is indicative of the intraneuronal trapping of MPP⁺.

Methods: Monkey BZ9 was injected with ³H₂-MPTP and killed 24 h later. The left half of the brain was regionally dissected and homogenates were prepared as described in the text. An extract containing the radioactivity was prepared by adding an equal volume of ethanol to a small aliquot (0.5 ml) of an aqueous homogenate, vortexing thoroughly, then centrifuging at 16,000g for 15 min. Samples (50 µl) of a representative extract were then injected into an HPLC system consisting of a 5 µ, 25 cm, C-18 reverse-phase column with a mobile phase of acetonitrile/100 mM sodium acetate (60:40, v/v) containing 0.1% triethylamine (final pH 5.6) at a flow rate of 1.0 ml min⁻¹; 44 fractions were collected at 15-s intervals and counted in 10 ml Aquasol (NEN) at an efficiency of 46%. Recovery of injected radioactivity was 100%, in all cases.

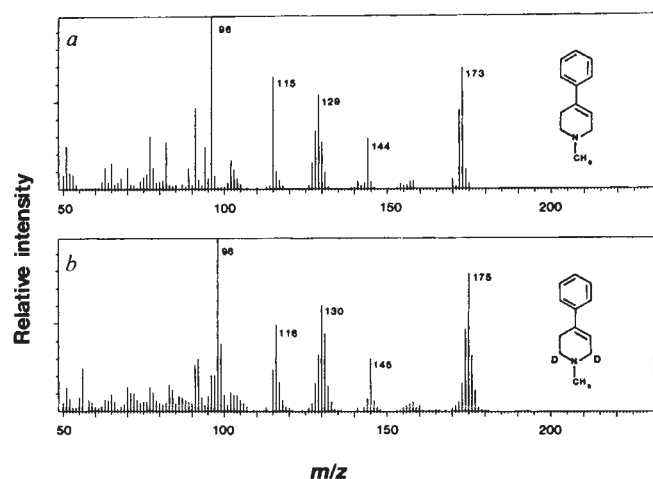


Fig. 2 *a*, Mass spectrum of authentic MPTP. *b*, Mass spectrum of metabolite reduced with NaB²H₄ which was extracted from brain tissue of a monkey treated with MPTP.

Methods: One-half of the brain of a rhesus monkey (tissue weight 39.1 g) which died 17 days after the last of five daily injections of MPTP (0.33 mg per kg per day, total dose 9.6 mg) was homogenized in 5 vol. water. This homogenate was extracted with an equal volume of ethanol and the protein precipitated by centrifugation at 23,000g for 20 min. The supernatant was washed three times with 2 vol. hexane, then reacted with 1.78 g of NaB²H₄ at room temperature for 30 min. The resulting product was extracted into hexane (two extractions with equal volumes of hexane), then concentrated to a volume of 5 ml by a series of forward and backward extractions of decreasing volume. A small quantity of decane (40 µl) was added to the final 5 ml of hexane, which was then evaporated to 40 µl under a gentle stream of N₂. This solution (0.5 µl) was injected into a 30 m × 0.32 mm DB-1 fused silica gas chromatographic column directly coupled to the ion source of a Finnigan 3200 quadrupole mass analyser.

(10 mg per kg); after 1 h both groups of mice were injected i.p. with MPTP (10 mg per kg) and 7.7 µCi [1-¹⁴C]MPTP. Pairs of mice (control and pargyline-pretreated) were killed by spinal dislocation 5, 10, 20, 30, 40, 60, 120 and 240 min after the latter injection. The brains were rapidly removed, frozen on dry ice, and stored at -20 °C until extracted and assayed by HPLC, as described in Fig. 3 legend. In a separate experiment, two groups of seven mice were treated as above, but injected with unlabelled MPTP alone, and killed 10 min later. The brains were removed and rapidly frozen, then stored until assayed by gas chromatography-mass spectrometry (GC-MS). Briefly, ²H₅- or ²H₁₀-internal standards were added to brain homogenates, basified, and MPTP extracted into toluene. Reducible metabolites were assayed as ²H₂-MPTP after reduction with sodium borodeuteride and toluene extraction. The organic extracts were derivatized with isobutyl chloroformate to form a stable derivative with excellent chromatographic properties, and assayed by determining the ratio of ions from unlabelled MPTP (*m/z* = 202), reducible metabolites (*m/z* = 204), and internal standards (*m/z* = 206 or 210).

The persistence of large amounts of radioactivity in the rhesus monkey brains was apparent from the percentages of administered radioactivity found in each brain (Table 1). In the case of monkey BZ9, 0.99% of the total injected dose was recovered from the brain after 24 h, whereas ~0.2% still remained after 20 days in monkey 28. From a log plot of the ³H data from four monkeys, a terminal-phase half life of 10 days was estimated. Virtually all of the radioactive material present in the monkey brains was ethanol-extractable from an aqueous tissue homogenate (98–99%). Measurements in 16 dissected brain regions from each monkey indicated that the pattern of radioactivity observed in the monkey brain at 24 h was consistent for at least 20 days. Furthermore, the use of a toxic dose did not affect the pattern observed with a tracer dose (for example, monkey BZ9 versus monkey 678).

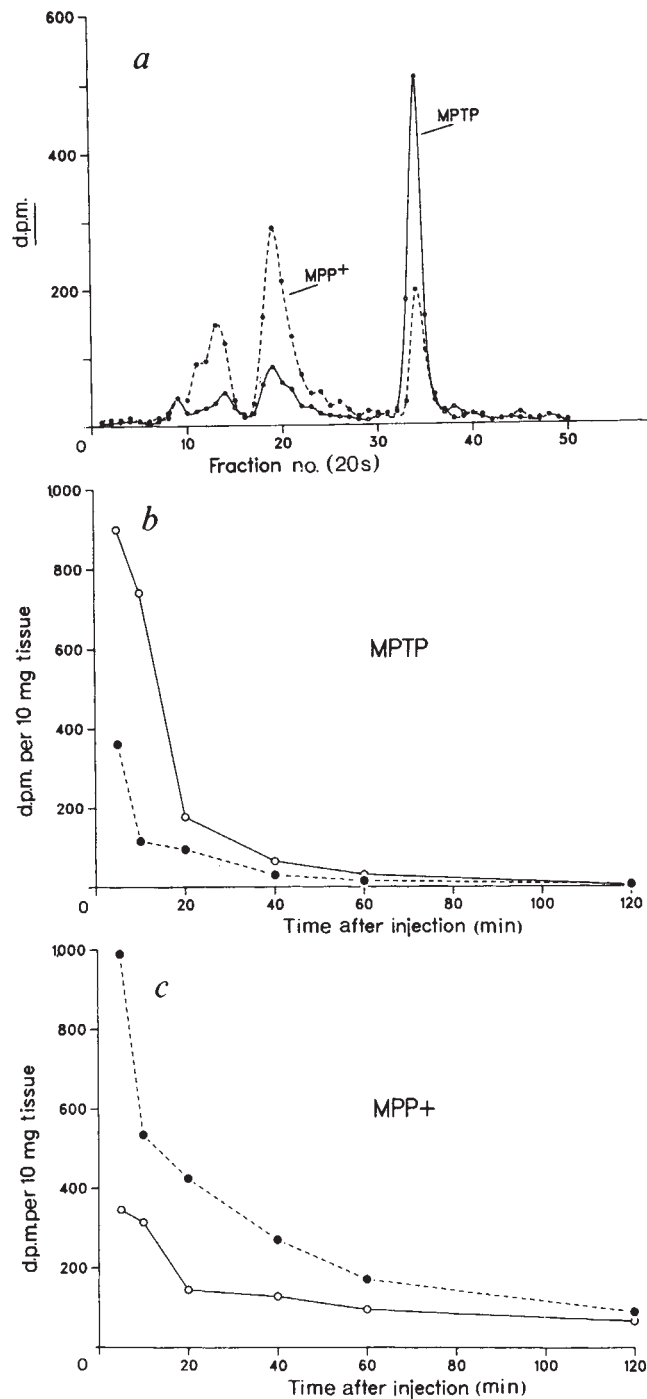


Fig. 3 Effect of pargyline pretreatment on MPTP metabolism in mice. ---●---, Control mice; —○—, pargyline-pretreated. The total radioactivity contained in the MPTP peak and MPP⁺ peak (*a*) was summed for each mouse brain extract and the totals plotted against the time after injection (*b* and *c*). Pargyline pretreatment increased the levels of MPTP at all times, while blocking the enzymatic conversion to MPP⁺.

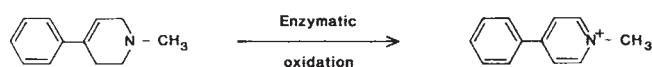
Methods: Fourteen adult male mice (NIH Swiss Webster, 20–22 g) were used in this experiment. Half were injected i.p. with 0.2 ml saline and half with 10 mg per kg pargyline in 0.2 ml saline; after 1 h all animals were injected i.p. with 0.2 ml of a saline solution containing 0.2 mg MPTP (10 mg per kg) and 7.7 µCi ¹⁴C-labelled MPTP. Pairs of mice (control and pargyline-pretreated) were killed by spinal dislocation 5, 10, 20, 40, 60, 120 and 240 min after the latter injection. The brains were rapidly removed, frozen on dry ice, and stored at -20 °C until used. Frozen brains were weighed, then homogenized in 4 vol. distilled water. A 0.5-ml aliquot of each homogenate was added to an equal volume of ethanol, vortexed, then centrifuged at 14,000g for 10 min. The radioactive components in the supernatant were separated by HPLC as described for Fig. 1.

Table 1 Amounts of radioactivity injected and survival times for four monkeys used in the study

Monkey	BZ9	910E	28	678
Weight (kg)	6.85	7.25	6.15	6.70
Dose of $^3\text{H}_2$ -MPTP (mCi per animal)	9.3	1.15	1.15	0.75
Dose of ^{14}C -MPTP (mCi per animal)	—	0.41	0.41	0.49
Total MPTP dose (mg per kg)	Trace	0.19	0.22	0.90
Survival time after injection (days)	1	10	20	5/10*
% Of injected ^3H in brain	0.99	0.32	0.18	0.47
% Of injected ^{14}C in brain	—	0.40	0.22	0.24

* $^3\text{H}_2$ -MPTP was injected 5 days after ^{14}C -MPTP. Total survival time was 10 days.

Figure 1b shows the HPLC analysis of a brain extract taken 24 h after a single injection of $^3\text{H}_2$ -MPTP (monkey BZ9). The elution profile remained constant regardless of either the brain region examined or the time since injection of MPTP. The major MPTP metabolite, which eluted as a single peak somewhat more polar than MPTP, always accounted for ~80% of the total radioactivity. To isolate and identify this metabolite, we considered expected routes of MPTP metabolism including *N*-demethylation, ring hydroxylation and protein alkylation⁷. The solubility properties of the monkey brain metabolite precluded protein alkylation. HPLC comparison of authentic *N*-dealkylated or 4-phenylhydroxylated standards indicated that the monkey brain metabolite is intermediate in polarity. We then considered the pyridinium oxidation product of MPTP, 1-methyl-4-phenylpyridine (MPP^+) (shown below).



Several lines of evidence have confirmed the presence of MPP^+ in the brains of monkeys treated with MPTP. First, formation of this product would require partial loss of ^3H from $[2,6\text{-}^3\text{H}_2]\text{MPTP}$, but would preserve the $1\text{-}^{14}\text{C}$ label, causing an increased $^{14}\text{C} : ^3\text{H}$ ratio. We observed this in monkeys which had received double-labelled MPTP (for example, the $^{14}\text{C} : ^3\text{H}$ ratio of the injection mixture used for monkey 28 was 0.36; in the caudate nucleus the ratio had increased to 0.68). Second, standards of MPP^+ co-chromatographed with the monkey brain metabolite (Fig. 1a). Treatment of radiolabelled monkey brain extract with NaBH_4 quantitatively converted the metabolite into a hexane-extractable base which co-chromatographed with MPTP (Fig. 1c). Finally, sufficient metabolite to analyse by GC-MS was isolated (see Fig. 2 for details) from the brain tissue of a monkey which died 17 days after treatment with MPTP (1.65 mg per kg). Reduction of the metabolite with NaBH_4 yielded a product determined by mass spectrometry to be $^2\text{H}_2$ -MPTP, the reduction product of the quaternary amine MPP^+ (Fig. 2). Thus, in monkey brain, MPTP is oxidized to MPP^+ , which is retained in the brain for over 20 days.

Our experiments in mice provide evidence that the metabolism of MPTP is, in fact, required for the expression of neurotoxicity. Heikkilä *et al.* have demonstrated that MPTP depletes neostriatal dopamine and destroys nerve cells in the zona compacta of the substantia nigra in mice⁴. This neurotoxicity can be blocked with monoamine oxidase (MAO) inhibitors including pargyline (see accompanying paper⁵). We found that pargyline decreased the oxidation of MPTP with a resultant increase in brain levels

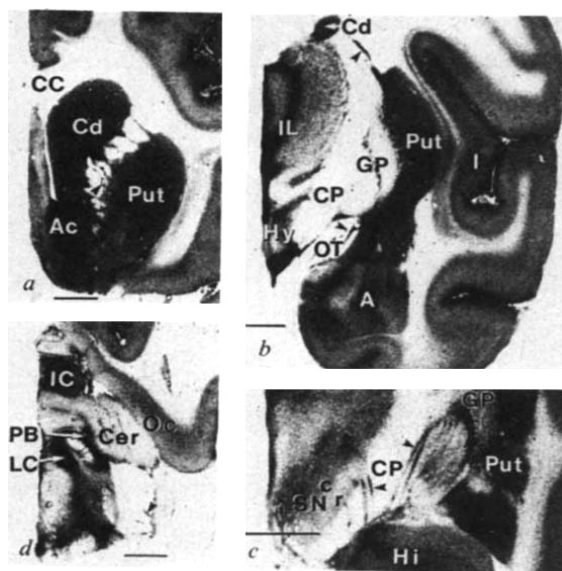


Fig. 4 Silver grain autoradiographic images of the brain of a monkey 24 h after treatment with $^3\text{H}_2$ -MPTP. Cryostat-cut brain sections (30 μm) from monkey BZ9 were thaw-mounted onto slides, dried quickly at 60 $^\circ\text{C}$ and apposed to ^3H -sensitive film (3H-Ultrofilm, LKB) for 5 weeks. After developing, the sections were counterstained with Thionine (not shown) to allow comparison of morphology with autoradiographic localization patterns. The films show that the walls of arteries accumulate the label densely (arrowheads indicate examples), white matter and the globus pallidus (GP)(b) very little, and other grey matter structures by varying amounts. Computer-assisted densitometry confirmed the relative radioactive densities derived from liquid scintillation counting of digests or extracts of dissected brain regions, and added further details. Densest labelling occurred in the locus coeruleus (LC) and lateral parabrachial nucleus (PB)(d); within the thalamus the intralaminar nuclei (IL)(b) were selectively dense; the entire neostriatum, including the n. accumbens (Ac), was moderately dense (a); the cerebral cortex showed lower and rather invariant levels of density; the substantia nigra had still lower density—the substantia nigra reticulata as low as the GP (c). A, amygdala; CC, corpus callosum; Cd, caudate; Cer, cerebellum; CP, cerebral peduncle; Hi, hippocampus; Hy, hypothalamus; I, insular cortex; IC, inferior colliculus; Oc, occipital cortex; OT, optic tract; Put, putamen; SN, substantia nigra (c, compacta; r, reticulata). Scale bar, 3 mm.

of MPTP and decreased concentrations of MPP^+ (Fig. 3). After 10 min, the ratio of MPTP to MPP^+ was 0.2 and 2.4 in the saline-pretreated and the pargyline-pretreated mouse brain, respectively, indicating the effectiveness of metabolic blockade. This observation was quantified in two groups of mice killed 10 min after injection with MPTP (10 mg per kg i.p.). Assay of MPTP and MPP^+ indicated higher levels of MPTP in the brains of pargyline-pretreated mice than in saline controls (188 ± 27 as opposed to 67 ± 13 ng per mg tissue $\pm$ s.e.m.; $P < 0.01$ using Student's *t*-test). Conversely, the MPP^+ levels were significantly lower in the brains of pargyline-pretreated than in saline-pretreated mice (63 ± 8 compared with 144 ± 17 ng per mg tissue; $P < 0.01$). Thus, pargyline blocks the oxidation of MPTP, but does not impair its entry to the brain. The fact that higher concentrations of MPTP were found at all times in the brains of pargyline-pretreated animals (Fig. 3b, c) indicates that MPTP cannot induce neurotoxicity without biotransformation. At times greater than 2 h, neither MPTP nor MPP^+ was present in appreciable amounts in the mouse brain, in marked contrast to the persistence of MPP^+ in the monkey brain. This difference in the persistence of MPP^+ may account for the difference in dose required to produce neurotoxicity in the mouse compared with the monkey (10 daily injections of 30 mg per kg produce only 80% neostriatal dopamine depletion in the mouse⁴ whereas in

the monkey, five daily injections of 0.33 mg per kg result in a 97% depletion³).

Oxidation of MPTP to MPP⁺ in the primate provides a facile intracellular trapping mechanism similar to that proposed by Bodor *et al.* for site-specific drug delivery of dihydropyridines⁸. Evidence for the intracellular trapping mechanism comes from several observations. First, the radioactivity in the monkey brain was not uniformly distributed, but correlated with cytoarchitecture as illustrated by autoradiographic images of the brain from monkey BZ9 (Fig. 4). High concentrations of radioactivity were observed in the caudate nucleus, putamen, hypothalamus, nucleus accumbens (n. accumbens) and the parabrachial nuclei, while low levels were seen in white matter (for example, corpus callosum) and globus pallidus. Second, the radioactivity in cerebrospinal fluid (CSF) taken from the ³H₂-MPTP-treated monkey (BZ9) at the time of killing was lower than in any brain tissue, and the HPLC profile showed a total absence of MPP⁺ (Fig. 1d). Finally, the MPP⁺ persisting in all of the treated monkeys was easily extracted into water on homogenization and remained in solution after protein precipitation, indicating trapping as opposed to covalent binding to intracellular proteins.

In the monkey, the localization of high concentrations of MPP⁺ in non-lesioned brain regions (for example, n. accumbens) suggests that the generation of MPP⁺ does not ensure cell death⁹. The properties of nigrostriatal dopamine neurones which render them more sensitive to the toxic effects of MPTP are unknown, but the selectivity may be dose- and time-dependent. Burns *et al.*³ clearly demonstrated temporary alterations in serotonergic and noradrenergic function following MPTP injection in the monkey.

We have demonstrated that MPP⁺ is formed from MPTP in both monkey and mouse brain. This process is necessary for the expression of neurotoxicity in the mouse as evidenced by decreased metabolic formation of MPP⁺ and increased MPTP concentrations in the brain in conditions which inhibit neurotoxicity. Whether MPP⁺ is a selective toxin or the oxidation process forming MPP⁺ is neurotoxic is unknown. The former notion is supported by the fact that MPP⁺ was previously developed as a selective herbicide (Cyperquat, Gulf Oil Company), although never marketed; it has been reported to have animal toxicity (50% lethal dose in rat, 35 mg per kg, oral)¹⁰. Alternatively, the dehydrogenation of MPTP may produce toxic intermediates (for example, free radicals, dihydropyridines, N-oxides, hydrogen peroxide) within nigrostriatal neurones, a mechanism proposed for other neurotoxins and for Parkinson's disease¹¹; this alternative is supported by the clinical observation that inhibition of monoamine oxidase by deprenyl prolongs the life of patients with idiopathic Parkinson's disease by slowing the disease process¹². Thus, there may be neurotoxic events common to both MPTP toxicity and Parkinson's disease. Regardless of whether it is the process or the product that is important, the study of the cellular consequence of the metabolic conversion of MPTP to MPP⁺ appears relevant to our understanding of the pathogenesis of Parkinson's disease.

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Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors

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1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) causes degeneration of the dopaminergic nigrostriatal pathway in several animal species, including humans^{1,2}, monkeys^{3,4} and mice⁵⁻⁷. Changes observed after MPTP administration include marked decrements in the neostriatal content of dopamine and its major metabolites, dihydroxyphenylacetic acid and homovanillic acid, and a greatly diminished capacity of neostriatal synaptosomes to take up ³H-dopamine^{5,6}. In contrast, there is no pronounced loss of serotonin in the neostriatum or of dopamine and its metabolites in other brain areas in MPTP-treated animals. The oxidative metabolism of MPTP to 1-methyl-4-phenyl pyridine, a positively charged species, has been suggested as a critical feature in the neurotoxic process⁸. Moreover, in rat brain preparations, the monoamine oxidase (MAO) inhibitor pargyline and the specific MAO-B inhibitor deprenil can prevent the formation of 1-methyl-4-phenyl-pyridine from MPTP, while the specific MAO-A inhibitor clorgyline has no such effect⁹, suggesting that MAO, and specifically MAO-B, is responsible for the oxidative metabolism of MPTP. We now report that pargyline, nialamide and tranlycypromine, which inhibit both MAO-A and MAO-B, when administered to mice before MPTP, protect against MPTP-induced dopaminergic neurotoxicity. Deprenil is also protective, but clorgyline is not. Our data are consistent with the premise that MAO-B has a crucial role in MPTP-induced degeneration of the nigrostriatal dopaminergic neuronal pathway.

Male, Swiss-Webster mice weighing 25–35 g were injected intraperitoneally with pargyline, deprenil, clorgyline, tranlycypromine or nialamide and subsequently received MPTP.

As Table 1 shows, 3 days after the last of five injections of MPTP, 30 mg per kg, the mean neostriatal content of dopamine (2.4 µg per g) fell markedly compared with the mean control value (10.7 µg per g). Animals given pargyline or nialamide alone had dopamine levels somewhat higher than control values, while the neostriatal dopamine content in mice receiving pargyline or nialamide before MPTP was similar to that in mice receiving the MAO inhibitors alone. The differences in dopamine levels between mice receiving MPTP alone and those receiving the MAO inhibitors plus MPTP were highly significant ($P < 0.001$). Thus, MPTP was non-toxic in the mice pretreated with either pargyline or nialamide. Similar results were obtained in mice killed 7 days after the last MPTP injection.

MPTP administration to mice also caused a pronounced decrement in the neostriatal content of the major metabolites of dopamine—dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)—which was evident at both 3 and 7 days after the last MPTP injection, as has previously been reported⁵⁻⁷. This effect was probably due to the neurotoxic effects of MPTP. Mice treated with pargyline or nialamide, alone or before MPTP, also showed marked reductions in neostriatal levels of DOPAC and HVA. This latter effect was probably due to the residual inhibition of MAO by pargyline and nialamide, both of which are known to cause a long-lasting and irreversible inhibition of MAO. DOPAC and HVA are both formed from the enzymatic actions of MAO on the ethylamine side chain of dopamine and their levels should thus be diminished as long as MAO activity is decreased¹⁰. Any protective effect of pargyline

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Table 1 The effects of pargyline or nialamide pretreatment on the MPTP-induced effects in mouse brain

MAOI	Treatment	Assay time (days)	n	Dopamine	DOPAC ($\mu\text{g per g tissue}$)	HVA	$^3\text{H-dopamine uptake}$ (fmol per mg tissue)	% Of control
—	—	—	8	10.7 ± 1.2	4.7 ± 0.9	2.3 ± 0.5	814 ± 227	100
Pargyline	—	3	3	13.3 ± 0.3	2.7 ± 0.4	1.6 ± 0.3	838 ± 50	103
Nialamide	—	3	3	14.5 ± 1.5	1.8 ± 0.3	1.2 ± 0.2	823 ± 99	101
—	MPTP	3	5	2.4 ± 0.8	1.8 ± 0.3	1.3 ± 0.3	360 ± 40	44
Pargyline	MPTP	3	5	13.0 ± 1.4	2.2 ± 0.3	1.4 ± 0.3	784 ± 80	96
Nialamide	MPTP	3	3	12.8 ± 1.1	1.5 ± 0.4	1.3 ± 0.3	764 ± 106	94
Pargyline	—	7	3	14.4 ± 1.1	2.2 ± 0.6	1.6 ± 0.3	765 ± 91	94
Nialamide	—	7	3	15.2 ± 1.2	1.8 ± 0.3	0.9 ± 0.1	645 ± 154	79
—	MPTP	7	4	3.5 ± 0.4	1.5 ± 0.6	1.2 ± 0.1	359 ± 60	44
Pargyline	MPTP	7	5	13.7 ± 1.1	2.1 ± 0.2	1.5 ± 0.2	750 ± 63	92
Nialamide	MPTP	7	4	10.5 ± 3.1	1.2 ± 0.2	1.0 ± 0.2	624 ± 175	77

Parameters studied were levels of dopamine and its metabolites in mouse neostriatum and the capacity of a mouse synaptosomal preparation to accumulate $^3\text{H-dopamine}$. The mice were treated with 15 mg per kg of pargyline or nialamide and subsequently given intraperitoneal injections of MPTP, 30 mg per kg (0.17 mmol per kg), dissolved in distilled water with the pH adjusted to 8.5 by dilute hydrochloric acid. Other mice received the MAO inhibitor alone, MPTP alone or no injections. Injection volumes were 1.0 ml per 100 g body weight. Levels of dopamine, serotonin and their metabolites were assayed in the neostriatum at various times after the last injection. The mice were stunned by a blow to the head, the brains removed and the neostriata rapidly dissected out. The paired neostriata were then weighed and homogenized in 0.3 M sucrose. An aliquot of this homogenate was diluted with an equal volume of 0.2 M perchloric acid containing dihydroxybenzylamine as an internal standard. The samples were centrifuged at 27,000g for 15 min and the supernatant used for assays¹¹. A Bioanalytical Systems LC-304T liquid chromatograph connected to a Kipp and Zonen dual pen recorder was used for the assays. The operating potential was 750 mV, the temperature 25 °C; full scale on the detector was usually set to 1.0 nA. A Biophase ODS 5 μm column (Bioanalytical Systems) was used for all separations, with a flow-rate for the mobile phase of 1.5 ml min⁻¹. The mobile phase was made up as follows: 35 ml of acetonitrile was added to 965 ml of 0.15 M monochloroacetate buffer at pH 3.0 containing 193 mg of sodium octyl sulphate. This mixture was filtered and degassed, tetrahydrofuran (18 ml) was carefully added, and the mobile phase capped until use. The samples were injected into the liquid chromatograph and data calculated from standard curves run the same day based on five or six data points in the same concentration range as that present in the tissue. Levels of dopamine and its major metabolites DOPAC and HVA, and serotonin and its major metabolite 5-hydroxyindoleacetic acid were calculated on the basis of the original tissue weight and the results expressed in $\mu\text{g per g of tissue} \pm \text{s.d.}$ In some experiments, in the remaining 0.3 M sucrose homogenate, a synaptosomal preparation was made and the uptake of 2 nM $^3\text{H-dopamine}$ (30.2 Ci mmol⁻¹, NEN) was measured as described previously¹². All data were calculated as fmoles of $^3\text{H-dopamine}$ accumulated per mg of tissue used $\pm \text{s.d.}$ and the data expressed as a per cent of control. Statistical analyses were done using Student's *t*-test.

or nialamide against MPTP, based on metabolic profiles, would therefore be masked by their capacity to inhibit MAO. These data indicate that decrements in levels of dopamine metabolites cannot by themselves always serve as a marker for determining whether toxicity to dopaminergic systems has occurred.

Table 1 also shows that neostriatal synaptosomes prepared from MPTP-treated mice had a greatly diminished capacity to take up $^3\text{H-dopamine}$ compared with control preparations. This observed decrement was the same (56%) at both 3 and 7 days after the last MPTP injection, indicating clearly that there was no recovery in MPTP-treated mice during this time^{6,7}. In contrast to this marked decrement in $^3\text{H-dopamine}$ uptake, the $^3\text{H-dopamine}$ uptake in neostriatal synaptosomal preparations from mice pretreated with pargyline or nialamide before MPTP was relatively close to or only slightly below control values (Table

1), as was $^3\text{H-dopamine}$ uptake in mice treated with pargyline or nialamide alone. The difference in $^3\text{H-dopamine}$ uptake between mice receiving MPTP alone and those receiving the MAO inhibitors plus MPTP were significant (*P* values ranged from <0.05 to <0.001).

Six injections of MPTP produced a very large fall in the neostriatal content of dopamine, evident at both 5 and 12 days after the last MPTP injection (Table 2). The differences in neostriatal dopamine content between control and MPTP-treated animals were highly significant (*P* < 0.001). MPTP treatment also caused large decrements in the neostriatal content of DOPAC and HVA (Table 2). The MAO-B inhibitor deprenil was completely protective against the MPTP-induced dopaminergic neurotoxicity (Table 2). Levels of dopamine and its metabolites in animals receiving deprenil plus MPTP did not

Table 2 The effects of deprenil or clorgyline pretreatment on the MPTP-induced decrements in the neostriatal levels of dopamine and its metabolites

MAOI	Treatment	Assay time (days)	n	Dopamine	DOPAC ($\mu\text{g per g tissue}$)	HVA
—	—	—	7	11.7 ± 1.1	3.6 ± 1.0	2.7 ± 0.5
Deprenil	—	5	4	13.1 ± 2.4	3.0 ± 0.4	2.8 ± 0.7
Clorgyline	—	5	4	13.8 ± 0.9	2.5 ± 0.6	1.6 ± 0.3
—	MPTP	5	5	2.7 ± 0.6	1.2 ± 0.3	1.3 ± 0.4
Deprenil	MPTP	5	5	13.2 ± 1.4	2.4 ± 0.3	2.4 ± 0.4
Clorgyline	MPTP	5	5	4.9 ± 2.6	1.0 ± 0.3	1.0 ± 0.2
Deprenil	—	12	3	12.6 ± 1.0	2.9 ± 0.7	2.3 ± 0.5
Clorgyline	—	12	3	12.7 ± 1.4	2.5 ± 0.4	1.8 ± 0.1
—	MPTP	12	3	2.9 ± 0.8	1.4 ± 0.2	1.5 ± 0.4
Deprenil	MPTP	12	6	12.6 ± 1.3	2.3 ± 0.4	2.1 ± 0.3
Clorgyline	MPTP	12	3	4.6 ± 2.6	1.3 ± 0.2	1.4 ± 0.2

The mice were treated with the MAO-inhibitor at 10 mg per kg on days 1 and 8, and with 30 mg per kg of MPTP on days 1, 2, 3, 8, 9 and 10. On days 1 and 8, the MPTP was given 30 min after the MAO-inhibitor. The mice were then killed either 5 or 12 days after the last MPTP injection (on days 15 and 22, respectively). The data represent the mean value $\pm \text{s.d.}$

differ significantly from levels in mice receiving deprenil alone ($P > 0.1$). However, in contrast to these protective effects of deprenil, the MAO-A inhibitor clorgyline, in identical conditions, failed to protect against MPTP-induced toxicity. Thus, dopamine levels were not significantly different in mice receiving MPTP alone and those given clorgyline plus MPTP ($P > 0.1$).

These data clearly demonstrate that two general MAO inhibitors, pargyline and nialamide (Table 1), and the specific MAO-B inhibitor deprenil (Table 2), effectively protect against the neostriatal dopaminergic neurotoxicity of MPTP in Swiss-Webster mice. We have obtained similar protective effects of these three MAO inhibitors against MPTP-induced toxicity in C57BL mice. In other experiments in Swiss-Webster mice, tranlycypromine, another inhibitor of both MAO-A and MAO-B, also protected against MPTP dopaminergic neurotoxicity. For example, neostriatal dopamine levels in mice receiving three MPTP injections of 30 mg per kg, with assays done 4 days after the last injection, were $4.4 \pm 1.8 \mu\text{g per g tissue}$ ($n = 7$). In contrast, mice pretreated with 15 mg per kg of tranlycypromine 30 min before the MPTP injections had a neostriatal dopamine level of $13.3 \pm 1.9 \mu\text{g per g}$ ($n = 6$). Neostriatal dopamine levels in control mice and in mice receiving only tranlycypromine were $12.3 \pm 1.3 \mu\text{g per g}$ ($n = 8$) and $14.0 \pm 0.4 \mu\text{g per g}$ ($n = 3$), respectively.

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Monoclonal antibodies against carbohydrate differentiation antigens identify subsets of primary sensory neurones

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Dorsal root ganglion (DRG) neurones transmit cutaneous sensory information from the periphery to the spinal cord. Within the dorsal horn of the spinal cord, classes of sensory fibres that are activated by different cutaneous stimuli terminate in separate and highly restricted laminae^{1–3}. Although the developmental events resulting in the laminar organization of sensory afferent terminals have not been defined, it is likely that interactions between surface molecules on DRG and dorsal horn neurones are involved in the generation of afferent synaptic connections. The identification of surface antigens that distinguish functional subclasses of DRG neurones would represent a first step in establishing the existence and nature of such molecules. We report here that monoclonal antibodies directed against carbohydrate differentiation antigens^{4,5} identify cytoplasmic and cell surface molecules expressed selectively by functional subsets of DRG neurones.

We used monoclonal antibodies to preimplantation murine embryos and human embryonal carcinoma cells, which recognize the developmentally-regulated stage-specific embryonic antigens, SSEA-3 and SSEA-4^{6–8}. These antigens have been characterized as overlapping carbohydrate epitopes on globo-series glycolipids⁸ (Table 1). Their presence in primary sensory neurones is demonstrated by indirect immunofluorescence on frozen or aldehyde-fixed sections of adult rat DRG and spinal cord. Incubation of DRG sections with anti-SSEA-3 or anti-SSEA-4 results in the labelling of neuronal cell bodies (Fig. 1a,

Our data are thus consistent with the premise that pargyline, nialamide, tranlycypromine and deprenil protect against the neurotoxic actions of MPTP by preventing the enzymatic formation of active metabolites of MPTP catalysed by MAO-B. However, it would be incautious to conclude that the prevention by these MAO inhibitors of the oxidative metabolism of MPTP is the sole mechanism for the observed protection. Nevertheless, the data have several important implications. First, they show that the mouse is an extremely useful small animal in which to study the mechanism of action of MPTP. Second, if the mouse data can be extrapolated to the monkey or human, a good working hypothesis would be that treatment with pargyline, nialamide, deprenil or other drugs capable of inhibiting MAO-B might counteract the neurotoxic actions of MPTP in the monkey or human. Third, and perhaps most important, if there is any relationship between the mechanism for the induction of MPTP-induced parkinsonism in experimental animals and Parkinson's disease in humans, investigators should be aware of a potential role for MAO in human parkinsonism.

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b) and sensory axons within the DRG and dorsal roots (Fig. 1a–d), with no detectable staining of non-neuronal elements. Intense SSEA-3 and SSEA-4 immunoreactivity occurs within the cytoplasm of DRG neurones and in association with the neuronal plasma membrane. Anti-SSEA-3 labels 9% and anti-SSEA-4 labels 11% of all DRG neurones (Table 2a), the majority of these being of intermediate and large diameter (30–60 μm). The percentage of immunoreactive neurones is constant in the DRG at cervical, thoracic and lumbar levels and there is no obvious topographical organization of labelled neurones within the DRG. SSEA-3 and SSEA-4 epitopes are also expressed by subpopulations of neurones in cranial sensory ganglia (Table 3), and are detected in subpopulations of DRG neurones in the mouse, cat and squirrel monkey (data not shown). A more complete description of SSEA distribution in primary sensory neurones will be reported elsewhere.

To determine the relationship between SSEA-3⁺ and SSEA-4⁺ DRG neurones in the rat, we used subclass-specific second antibodies that distinguish anti-SSEA-3 (a rat IgM) from anti-SSEA-4 (a mouse IgG3) on single sections of the DRG. Most immunoreactive DRG neurones are SSEA-3⁺/SSEA-4⁺ (Fig. 1f, g; Table 2a); a few small-diameter neurones are, however, solely SSEA-3⁺ and many large-diameter neurones are solely SSEA-4⁺ (Table 2a). Double labelling has shown that SSEA-3⁺, SSEA-3⁺/SSEA-4⁺ and SSEA-4⁺ neurones are distinct from the DRG neurones that express the peptide transmitters substance P and somatostatin⁹. Sequential immunofluorescence and histochemical staining demonstrate that SSEA-3⁺, SSEA-3⁺/SSEA-4⁺ and SSEA-4⁺ neurones are also distinct from those that express the fluoride-resistant acid phosphatase isoenzyme (FRAP)^{10,11}.

Table 1 Stage-specific embryonic antigen carbohydrate structure

Antibody	Epitope	Immunogen	Ref.
Anti-SSEA-3	GalNAc β 1 $\rightarrow$ 3 Gal α 1 $\rightarrow$ 4Gal	4–8 cell stage murine embryos	7, 8
Anti-SSEA-4	NeuAc α 2 $\rightarrow$ 3 Gal β 1 $\rightarrow$ 3GalNAc	2102Ep teratocarcinoma	8

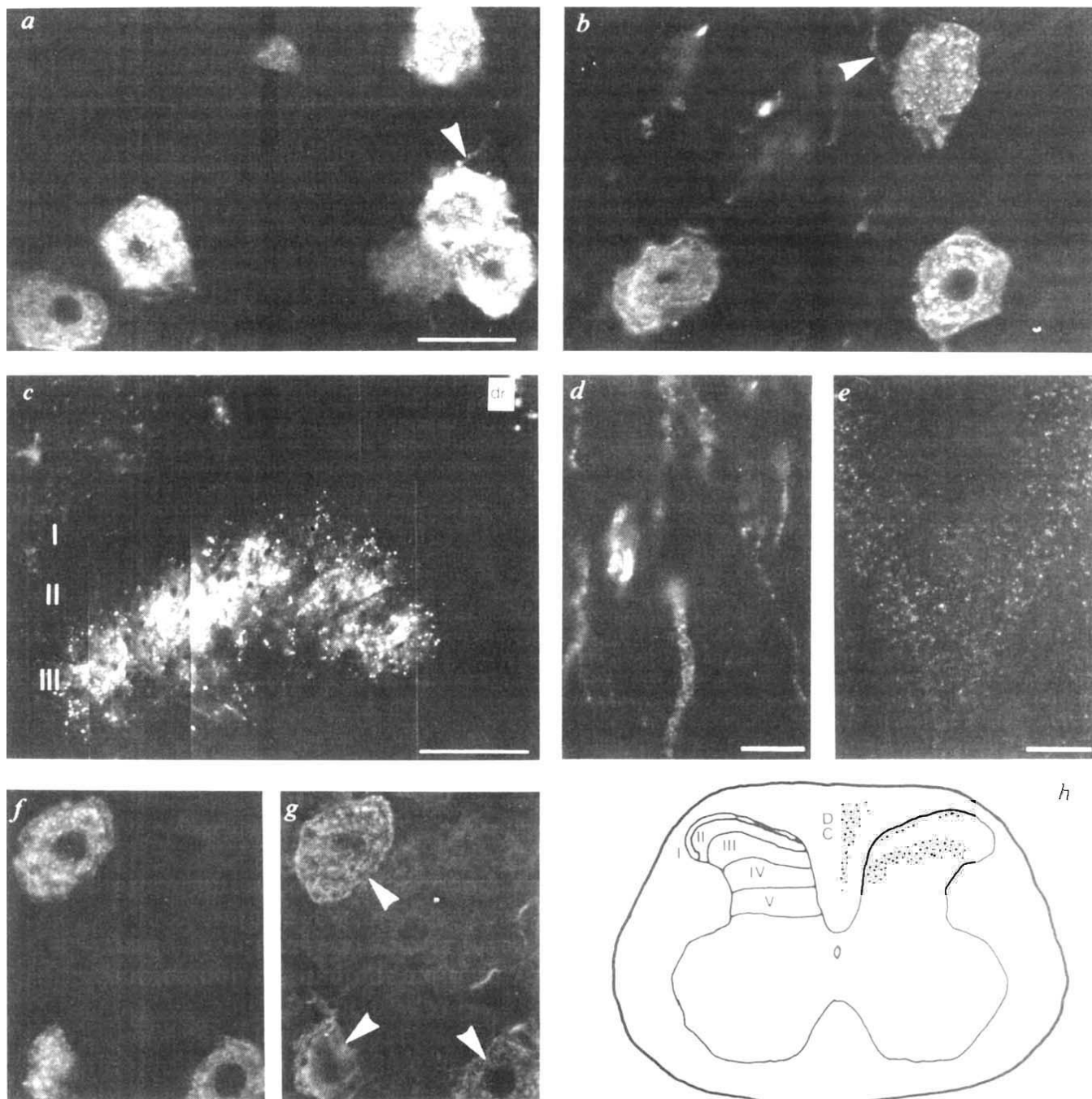


Fig. 1 Expression of SSEA epitopes by subpopulations of primary sensory neurones. Anti-SSEA-3 (a) and anti-SSEA-4 (b) produced bright, cytoplasmic labelling of a subpopulation of neurones in sections of DRG (arrows indicate stained axons). c, SSEA-4⁺ fibres and terminals are found in a dense band in lamina III of the dorsal horn, with scattered fibres projecting into lamina IV. Immunoreactive afferent fibres can also be seen in cross-section, overlying the medial dorsal horn. In addition, SSEA-4⁺ afferents are present in the dorsal root (dr) seen here in cross-section. In c and h, I, II, III, IV and V represent the location of the Rexed's laminae. d, SSEA-3⁺ fibres are shown in a longitudinal section through a dorsal root. e, The distribution of SSEA-3⁺ fibres in a transverse section through the dorsal columns. f, g, A single section of DRG was incubated with anti-SSEA-3 and anti-SSEA-4 and the distribution of the two antibodies was visualized using subclass-specific antibodies coupled to different fluorophores. f, Three DRG neurones that express SSEA-3; g shows the presence of SSEA-4 in the same neurones (arrows). Control experiments indicated that neither cross-reactivity of the antibodies nor barrier filter leakage contributed to this result. h, A diagram of a coronal section of the rat spinal cord, shows the laminar distribution of the central processes of SSEA-3⁺ and SSEA-4⁺ DRG neurones. SSEA-3⁺ and SSEA-4⁺ fibres were found predominantly in lamina III with a sparse representation in lamina I, a dense region of staining at the medial border of laminae III and IV and stained fibres in the cuneate and gracile fasciculi (DC). Scale bars: a, b, f, g, 40 μ m; c, 80 μ m; d, 10 μ m; e, 150 μ m.

Methods: Rat tissues were fixed by cardiac perfusion with 50 ml ice-cold saline followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Cryostat sections (10 μ m) were incubated with anti-SSEA ascites fluid, diluted 1:100 to 1:300 in phosphate-buffered saline (PBS: 0.1 M PB in 0.9% saline) containing 10% fetal calf serum and 0.2% Triton X-100, pH 7.2 (16 h; 4°C). a–e, Sections were then washed with PBS containing 2% normal goat serum (PBS/NGS), incubated with fluorescein isothiocyanate (FITC) goat anti-mouse IgM or IgG (Tago, Inc. and Boehringer-Mannheim, respectively) diluted 1:75 in PBS/NGS for 30 min at room temperature. Sections were then washed in PBS/NGS and coverslipped in 50% glycerol in 0.1 M sodium carbonate buffer, pH 9.0, containing 0.4 mg ml⁻¹ p-phenylenediamine (Sigma). f, g, Following incubation with anti-SSEA, sections were washed in PBS/NGS and incubated in PBS/NGS containing FITC-goat anti-mouse IgM, 1:75 and unconjugated rabbit anti-mouse IgG3, 1:30 (Litton Bionetics), for 30 min at room temperature. Sections were then washed again in PBS/NGS and incubated with FITC-goat anti-mouse IgM, 1:75 plus rhodamine-goat anti-rabbit IgG, 1:200 (Tago), for 30 min at room temperature. Sections were then washed and coverslipped as described above.

Table 2 Expression of SSEAs by rat DRG neurones

<i>a</i> , Cytoplasmic staining in adult rat DRG neurones <i>in situ</i>		
Antigen	% Immunoreactive neurones	% Overlap
SSEA-3	9 (<i>n</i> = 7868)	68 SSEA-4 ⁺
SSEA-4	11 (<i>n</i> = 4789)	55 SSEA-3 ⁺
<i>b</i> , Surface staining of DRG neurones in culture		
Antigen	% Immunoreactive neurones	% Overlap
SSEA-3	7 (<i>n</i> = 1060)	84 SSEA-4 ⁺
SSEA-4	14 (<i>n</i> = 1775)	40 SSEA-3 ⁺

Table 3 Expression of SSEAs in primary sensory neurones

	% SSEA-3	% SSEA-4
Dorsal root ganglion	9	11
Trigeminal ganglion	20	35
Petrosal ganglion	15	15
Jugular ganglion	<5	10
Nodose ganglion	60	>90

The distribution of molecules bearing the SSEA-3 and SSEA-4 epitopes in the central nervous system was examined by indirect immunofluorescence on 10 µm coronal sections taken at 100 µm intervals from the caudal brain stem to the olfactory bulb. We do not find SSEA-3⁺ or SSEA-4⁺ neuronal cell bodies within the CNS. In the spinal cord and brain stem, SSEA-3⁺ and SSEA-4⁺ immunoreactive fibres and terminals are restricted to regions that receive primary afferent input. In the dorsal horn of the spinal cord, a few labelled fibres and terminals are found in lamina I (Fig. 1*h*), whereas the tract of Lissauer and lamina II are devoid of immunoreactive fibres (Fig. 1*c, h*). The greatest density of labelled fibres occurs in lamina III (Fig. 1*c*) and the medial region of lamina IV (Fig. 1*h*). SSEA-3⁺ and SSEA-4⁺ fibres are also present in the dorsal columns (Fig. 1*e*) and in the gracile and the external cuneate nuclei. No immunoreactive fibres are observed on the lesioned side of the spinal cord in animals unilaterally deafferented 3 weeks previously by section of the dorsal roots. These results indicate that all SSEA-3⁺ and SSEA-4⁺ fibres within the dorsal horn of the spinal cord derive from sensory neurones within DRG.

The precise laminar arrangement of the central terminals of cutaneous afferent fibres in the rat has not been mapped in detail. The laminar cytoarchitecture and the distribution of peptide-containing sensory terminals in the rat dorsal horn are, however, similar to those in cat and primate^{12,13}, and we have found the laminar distribution of SSEA-3⁺ and SSEA-4⁺ that fibres in the dorsal horn is similar in rat, cat and squirrel monkey (J.D., T.M.J. and E. R. Perl, unpublished observations). In cats and primates, the terminal arbors of several classes of low threshold afferents are known to overlap within laminae III and IV¹. Our observations are therefore consistent with the presence of SSEA-3 and SSEA-4 epitopes on low-threshold hair follicle afferents and mechanoreceptors^{14,15}. The absence of labelled terminals in lamina II indicates that unmyelinated primary afferents do not express the SSEA-3 and SSEA-4 epitopes.

The SSEA-3 and SSEA-4 carbohydrate sequences are also expressed on the surface of DRG neurones. Approximately 7% of neonatal rat DRG neurones maintained in dissociated cell culture exhibit SSEA-3 immunoreactivity on neuronal somata and processes (Fig. 2*a, b*; Table 2*b*), and the SSEA-4 epitope is present on 14% of cultured DRG neurones (Fig. 2*c, d*; Table 2*b*). Dual-colour labelling with subclass-specific second antibodies reveals that the relationship between SSEA-3 and SSEA-4 epitopes on the surface of cultured DRG neurones is similar to that observed in the cytoplasm of DRG neurones *in situ*: over 80% of SSEA-3⁺ DRG neurones in culture are SSEA-4⁺ and, conversely, 40% of SSEA-4⁺ neurones are SSEA-3⁺ (Table 2*b*). SSEA-3⁺ and SSEA-4⁺ DRG neurones may correspond to the

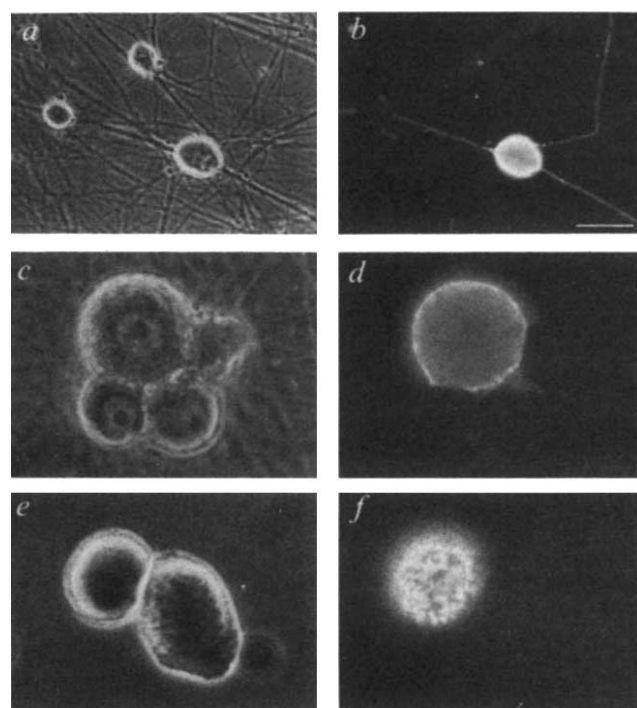


Fig. 2 Expression of SSEA epitopes on the surface of cultured DRG neurones. *a*, Phase micrograph showing three DRG neurones and a plexus of neuronal processes. *b*, Fluorescence micrograph of the field shown in *a*: one of the three neurones expresses intense SSEA-3 immunoreactivity on its cell soma and processes. *c*, Phase-contrast micrograph showing four DRG neurones. *d*, Fluorescence micrograph of the field shown in *c*: one of the neurones expresses SSEA-4 immunoreactivity. *e*, Phase-contrast micrograph of two adult rat DRG neurones. *f*, Immunofluorescent micrograph of the field shown in *e*, in which only one neurone expresses intense SSEA-3 immunoreactivity. Scale bar: *a, b*, 30 µm; *c, d*, 20 µm; *e, f*, 20 µm.

Methods: DRG neurones were isolated from neonatal rats and maintained in dissociated cell culture for periods of 14–42 days using previously described methods¹⁶. Non-neuronal cells were eliminated from DRG cultures by treatment with cytosine arabinoside (10 µM) for 24–48 h soon after plating. Live cultures were washed in HEPES-buffered balanced salt solution (BSS) supplemented with glucose and 0.1% bovine serum albumin. Cultures were then incubated with anti-SSEA ascites fluid diluted 1:100 to 1:200 in buffered BSS containing 1% NGS. FITC-labelled second antibodies were diluted in buffered BSS and used as described in the legend to Fig. 1.

population of cultured sensory neurones that are labelled with antiserum to the globo-series glycolipid, globoside¹⁶.

We excluded the possibility that the cell surface expression of SSEA-3 and SSEA-4 epitopes is a result of maintaining DRG neurones in culture, by labelling adult rat DRG neurones immediately after dissociation. Many of these neurones are also SSEA-3⁺ and SSEA-4⁺ (Fig. 2*e, f*), but the low cell yields obtained after mechanical dissociation of adult DRG neurones preclude determination of the percentage of labelled cells.

The SSEA-3 and SSEA-4 epitopes are associated with glycolipids and glycoproteins on murine embryos and human embryonal carcinoma cells^{6,8}. SSEA-3 and SSEA-4 immunoreactivity in fixed sections of DRG and spinal cord is abolished by chloroform-methanol treatment (1:1, 15 min) whereas peptide and protein antigens, recognized by other monoclonal antibodies, are not affected by identical treatment. Moreover, preincubation of sections with 10 mM sodium periodate for 30 min at room temperature, substantially reduced SSEA-3 and SSEA-4 immunoreactivity. These observations suggest that in sensory neurones, both epitopes are associated, at least in part, with glycolipids.

Our results suggest that subpopulations of DRG neurones involved in the processing of low threshold cutaneous stimuli can be identified by the surface expression of globo-series carbohydrates. In preliminary experiments, we have detected the SSEA-3 and SSEA-4 epitopes on some DRG neurones in 16-day rat embryos, soon after final mitosis¹⁷. Therefore, anti-SSEA antibodies may provide specific probes to examine early development and differentiation of this class of DRG neurones. Several other monoclonal antibodies have recently been generated against carbohydrate sequences expressed on the surface of embryonic or transformed cells^{18,19}. We have found that some of these carbohydrate determinants are expressed selectively on subsets of DRG neurones distinct from those that express SSEA-3 and SSEA-4 epitopes²⁰, suggesting that many subclasses of DRG neurones may be characterized by differences in cell surface carbohydrate expression.

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Cell surface carbohydrates have been implicated in intercellular adhesion and recognition in early embryos and embryonal carcinoma cells^{21–23}, and in neuronal adhesion during embryonic development^{24,25}. Molecules bearing SSEA-3, SSEA-4 and other carbohydrate determinants could therefore be involved in the development and specification of sensory connections in the dorsal horn of the spinal cord.

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Pattern of presynaptic nerve activity can determine the type of neurotransmitter regulating a postsynaptic event

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The mammalian superior cervical ganglion has been the classical preparation for studying cholinergic transmission between neurones^{1–3}. Recently, however, evidence has been presented showing that, in addition to the postsynaptic changes mediated via nicotinic and muscarinic receptors, there is a non-cholinergic component to transmission in this ganglion^{4,5}, as in frog paravertebral ganglia^{6,7}. In the rabbit superior cervical ganglion, Ashe and Libet recorded a late, slow excitatory postsynaptic potential in response to preganglionic nerve stimulation in the presence of nicotinic and muscarinic antagonists⁴. We have found, in the rat superior cervical ganglion, that a postsynaptic biochemical consequence of preganglionic nerve stimulation, namely the acute activation of tyrosine 3-monooxygenase (tyrosine hydroxylase, TH; EC 1.14.16.2), is mediated in part by acetylcholine and in part by a non-cholinergic neurotransmitter⁵. The regulation of this enzyme activity is of particular interest because it catalyses the rate-limiting step in the biosynthesis of the postganglionic neurotransmitter, noradrenaline. In the present paper, we report that the relative importance of cholinergic and non-cholinergic transmission in the regulation of TH activity varies with the pattern of electrical stimulation of the preganglionic nerve trunk.

Male Sprague Dawley rats (160–200 g) were used in all studies. The rats were killed by cervical dislocation and their superior cervical ganglia rapidly removed, desheathed and maintained *in vitro* at 37 °C as previously described⁵. The preganglionic nerve trunk of one ganglion was stimulated via a

suction electrode, and the effectiveness of the stimulation was monitored throughout the stimulation period by recording, via a second suction electrode, the compound action potentials elicited in the postganglionic internal carotid nerve. Except where noted, the current intensity used was twice that required to give a maximum compound action potential. Contralateral control ganglia from the same animals were maintained in similar conditions but were not stimulated. To initiate the measurement of the rate of tyrosine hydroxylation, brocresine (150 µM), an inhibitor of dopa decarboxylase, was added to the incubation medium 2 min before stimulation was begun. At the end of the stimulation period, stimulated and control ganglia were individually homogenized. The respective incubation media were added to the homogenates, and the combined samples were chilled on ice and then centrifuged for 2 min in a Beckman Microfuge. The supernatants were stored at –20 °C before determination of their dopa content by high performance liquid chromatography with electrochemical detection as described elsewhere^{5,8}. We have shown previously (1) that dopa is not detectable in ganglia incubated in the absence of brocresine, (2) that dopa accumulation in the presence of brocresine is blocked by 3-iodo-tyrosine, an inhibitor of TH, and (3) that dopa, once accumulated, is stable under our incubation conditions for at least 30 min. Thus dopa accumulation is a valid measure of tyrosine hydroxylation in this preparation⁸.

Preganglionic nerve stimulation at 10 Hz for 30 min resulted in about a 5-fold increase in dopa synthesis (Fig. 1). Addition of the nicotinic antagonist, hexamethonium (3 mM) and the muscarinic antagonist, atropine (6 µM) to the incubation medium inhibited the increase in dopa synthesis by 50% (Fig. 1). The concentration of hexamethonium used completely blocked the compound action potentials normally recorded during preganglionic nerve stimulation (data not shown), and the concentration of atropine used has been shown by others to block the muscarinic postsynaptic electrophysiological responses elicited by such stimulation^{9,10}. However, in the presence of both of these antagonists, nerve stimulation still produced a significant (approximately 3-fold) increase in dopa synthesis. When the preganglionic nerve was stimulated with 10 Hz bursts (10 Hz for 1 out of every 6 s) for 30 min, dopa

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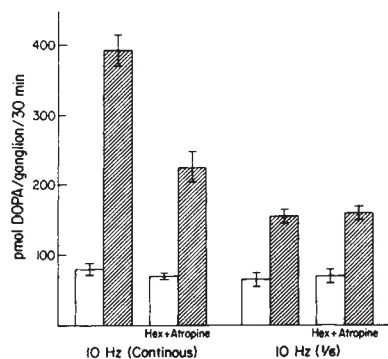


Fig. 1 Effect of cholinergic antagonists on the increase in dopa synthesis produced by preganglionic nerve stimulation. Ganglia were stimulated for 30 min either at 10 Hz continuously or at 10 Hz for 1 out of every 6 s. Hexamethonium (Hex, 3 mM) and atropine (6 μ M) were added to certain preparations 10 min prior to the beginning of stimulation. The data represent the mean $\pm$ s.e.m. of 4–6 ganglia. Control ganglia, open bars; stimulated ganglia, hatched bars.

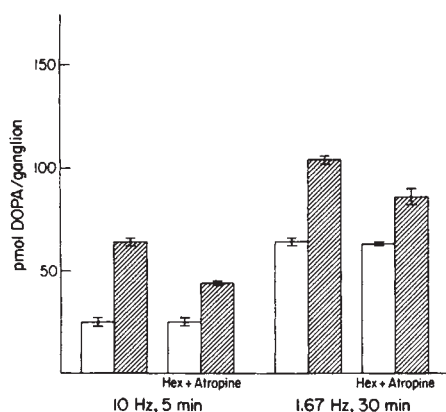


Fig. 2 Evidence for cholinergic and non-cholinergic regulation of tyrosine hydroxylase activity during continuous preganglionic nerve stimulation. Ganglia were stimulated at 10 Hz for 5 min or at 1.67 Hz for 30 min. Hexamethonium (Hex, 3 mM) and atropine (6 μ M) were added to certain preparations 10 min before stimulation. The data represent the means $\pm$ s.e.m. of 4–5 ganglia. Control ganglia, open bars; stimulated ganglia, hatched bars.

synthesis was elevated by 2.4-fold (Fig. 1). Under these conditions, the increase in dopa production was not lessened at all by the addition of hexamethonium and atropine (Fig. 1). On the other hand, the compound action potentials were blocked by these antagonists under both stimulation conditions (data not shown).

The pharmacology of the acute transsynaptic regulation of TH differs under these two stimulation conditions. These results indicate that the increase in TH activity following continuous stimulation at 10 Hz is partially mediated by acetylcholine and partially by a non-cholinergic transmitter, as we have previously reported⁵. However, the increase in TH activity produced by stimulation with 10 Hz bursts is mediated entirely by a non-cholinergic mechanism. The two patterns of nerve stimulation examined differ in two respects; that is, in the total number of pulses delivered (which is six times greater in the continuous pattern) and in the temporal distribution of the pulses. The effects of two other stimulation conditions were examined to determine if one of these factors is particularly important in determining whether or not a cholinergically mediated stimulation of dopa synthesis occurs. Under each of these conditions, the same number of pulses was delivered as in the 10 Hz burst pattern. Preganglionic nerve stimulation at 10 Hz for 5 min increased dopa synthesis by 2.5-fold (Fig. 2). This effect of nerve stimulation was decreased by 43% by adding hexamethonium and atropine (Fig. 2). Continuous stimulation at 1.67 Hz for

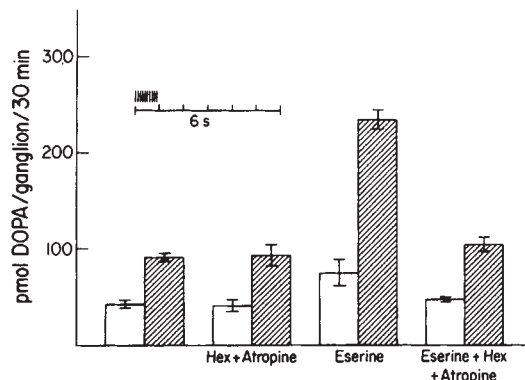


Fig. 3 Effects of physostigmine (eserine) and cholinergic antagonists on the increase in dopa production following "discontinuous" stimulation. Ganglia were stimulated at 10 Hz for 1 out of every 6 s for 30 min. Eserine (20 μ M) and/or hexamethonium (Hex, 3 mM) and atropine (6 μ M) were added to certain preparations 10 min before stimulation was begun. The current intensity used was that required to give a maximum compound action potential. In this experiment, the incubation medium used was Earle's balanced salt solution. In all other experiments reported in this paper, Kreb's bicarbonate buffer was used. The data represent the mean $\pm$ s.e.m. of 3–5 ganglia. Control ganglia, open bars; stimulated ganglia, hatched bars. The stimulation pattern is shown diagrammatically at the top of the figure.

30 min led to a smaller (62%) increase in dopa synthesis, which was reduced by 42% by the addition of the cholinergic antagonists (Fig. 2).

These data suggest that the temporal distribution, rather than the number, of impulses is the important factor in determining the occurrence of a cholinergically mediated increase in TH activity. Perhaps preganglionic nerve stimulation for brief periods (for example 1 s) followed by prolonged periods of inactivity (for example 5 s) does not provide a sustained enough cholinergic stimulation of the postganglionic neurones to elevate TH activity. Acetylcholine is rapidly cleared from the synaptic region following a nerve impulse^{3,11}, due to activity of the enzyme acetylcholinesterase and to diffusion away from the synaptic cleft. Such a rapid removal of acetylcholine may be essential for its role in transmitting electrophysiological information as the continuous presence of a cholinergic agonist can lead to desensitization of the action potential triggering mechanism in sympathetic ganglia, as it does at the neuromuscular junction¹². However, a brief exposure to an agonist, although suitable for triggering action potentials, may not be suitable for a sustained activation of an enzyme like TH. To investigate whether the degradation of acetylcholine prevents the expression of a cholinergic component in the regulation of TH during discontinuous nerve stimulation, ganglia were stimulated with 10 Hz bursts in the presence or absence of the acetylcholinesterase inhibitor, physostigmine. In agreement with the data reported in Fig. 1, stimulation with this discontinuous pattern produced about a 2-fold increase in dopa synthesis, which was completely resistant to cholinergic antagonists (Fig. 3). Addition of physostigmine (20 μ M) to the incubation medium significantly elevated the rate of dopa synthesis in both control and stimulated ganglia (Fig. 3). These effects of physostigmine were completely blocked by hexamethonium and atropine (Fig. 3). Physostigmine thus causes the expression of a cholinergic component of TH regulation during discontinuous nerve stimulation, either by increasing the amount of acetylcholine in the synaptic cleft, or by prolonging the duration of cholinergic stimulation or by both.

We do not know yet which transmitter is responsible for the non-cholinergic elevation of TH activity. So far, after screening a large number of peptides and other transmitter candidates, we have found that secretin, vasoactive intestinal peptide (VIP) and PHI (which refers to a 27-amino acid peptide that has a histidine at the N-terminal and an isoleucine amide at the

C-terminal) can mimic this non-cholinergic effect of nerve stimulation^{13,14}. Many other peptides tested, including glucagon, substance P, methionine-enkephalin, luteinizing hormone releasing hormone, and angiotensin had no effect on TH activity. The idea that certain neuropeptides may function as neurotransmitters in specific autonomic ganglia is now widely accepted¹⁵. Although we have not yet established that the non-cholinergic elevation of TH activity produced by preganglionic nerve stimulation is mediated by a neuropeptide, it is interesting to note that one characteristic of the electrophysiological effects of peptides, which has been commonly observed, is their long duration (for example, changes in resting potential lasting for a minute or longer, see refs 7, 16 for examples). Such a time course is compatible with our finding that the non-cholinergic (but not the cholinergic) regulation of dopa synthesis is present during a bursting pattern of nerve stimulation. Thus the postsynaptic consequences of a neuropeptide, unlike that of acetylcholine, might be sustained throughout the interburst interval. The magnitude of peptidergic postsynaptic effects may also be affected by the pattern of nerve stimulation. Thus, in certain peptidergic neurones, such as the VIP-containing parasympathetic neurones innervating the cat submandibular gland and the vasopressin- and oxytocin-containing neurones of the hypothalamo-hypophyseal system, the release of peptides is greater when impulses are delivered in high frequency bursts, rather than continuously at the same average frequency^{17,18}. Perhaps a similar mechanism accounts for the fact that while the non-cholinergic component of TH regulation is present during continuous stimulation at a low frequency (1.67 Hz), its magnitude appears to be enhanced by delivering the same number of impulses intermittently in bursts at a higher frequency (10 Hz for 1 out of every 6 s) (Figs 1 and 2). Significantly, studies on the physiological discharge pattern in sympathetic neurones have demonstrated that the electrical activity *in vivo* often occurs in brief bursts separated by periods of inactivity^{3,19}. Perhaps, in certain conditions (for example certain types of stress) a more continuous pattern of preganglionic nerve activity occurs, thus activating a second (cholinergic) mechanism for elevating noradrenaline synthesis.

Recent studies on the colon demonstrate that the nature of the tissue response to nerve stimulation (that is, whether vasodilation or contraction of the colon occurs) is dramatically altered by changing the pattern of stimulation²⁰. These responses may well be mediated by different neurotransmitters. In the present study, we have found that the pattern of stimulation determines the relative importance of different transmitters in regulating a single postsynaptic response, namely the stimulation of catecholamine biosynthesis.

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Giant synaptosomes

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Investigations using synaptosomes, pinched-off nerve ending particles from brain^{1,2}, have greatly improved our knowledge of presynaptic function. However, these structures, like most nerve endings, are too small to be penetrated with microelectrodes. We have treated synaptosomal preparations from rat brain with a neutral protease and obtained fused structures large enough to be recorded from directly with microelectrodes; we report here that these particles (30–250 μ M in diameter) contain mitochondria and structures resembling synaptic vesicles, morphological features characteristic of synaptosomes. These 'giant synaptosomes' have resting membrane potentials in the range -45 to -76 mV, are depolarized by increasing concentrations of K^+ , show responses to a variety of neuroactive substances and exhibit active membrane responses to depolarizing current pulses. These results suggest that this preparation will be of value in further studies of nerve terminal function.

Synaptosomal fractions were prepared from the brains of Wistar rats (25–100 g) using differential and sucrose gradient centrifugation as described¹, except that the initial homogenization step was performed by hand. The synaptosomal pellet from one brain was dispersed in approximately 0.5 ml of sucrose (0.32 M); neutral protease (dispace, Boehringer-Mannheim) was then added to a concentration of 0.1–0.2 mg ml⁻¹. The mixture was incubated for 30–40 min at 20–23 °C and samples were examined for fused synaptosomes using a light microscope. The number of giant particles formed (Fig. 1a) was in the range of 0–4 per preparation of synaptosomes. Giant particles failed to form in about half of the preparations.

Electron microscope investigation of these particles is difficult, mainly because of the low success rate of their formation. Nevertheless, the interior of the particles is nearly devoid of electron-dense material (Fig. 1b, c). Clustered along the limiting membrane there are mitochondria and occasional vesicles or clusters of vesicles of 40–60 nm diameter. Of the 10 particles examined in this way, none has contained ribosomes, rough endoplasmic reticulum or nuclei, which suggests that cell bodies do not participate in the genesis of these structures. The 40–60 nm vesicles could be synaptic vesicles, which along with mitochondria, are features typical of synaptosomes¹. Also consistent with the interpretation that these giant particles derive from synaptosomes is the fact that similar structures have not been obtained either using the P₂ (crude mitochondrial fraction of Gray and Whittaker¹) or the myelin or mitochondrial fractions from the sucrose gradients used to isolate the synaptosomes. Provisionally, we will call the fused structures giant synaptosomes.

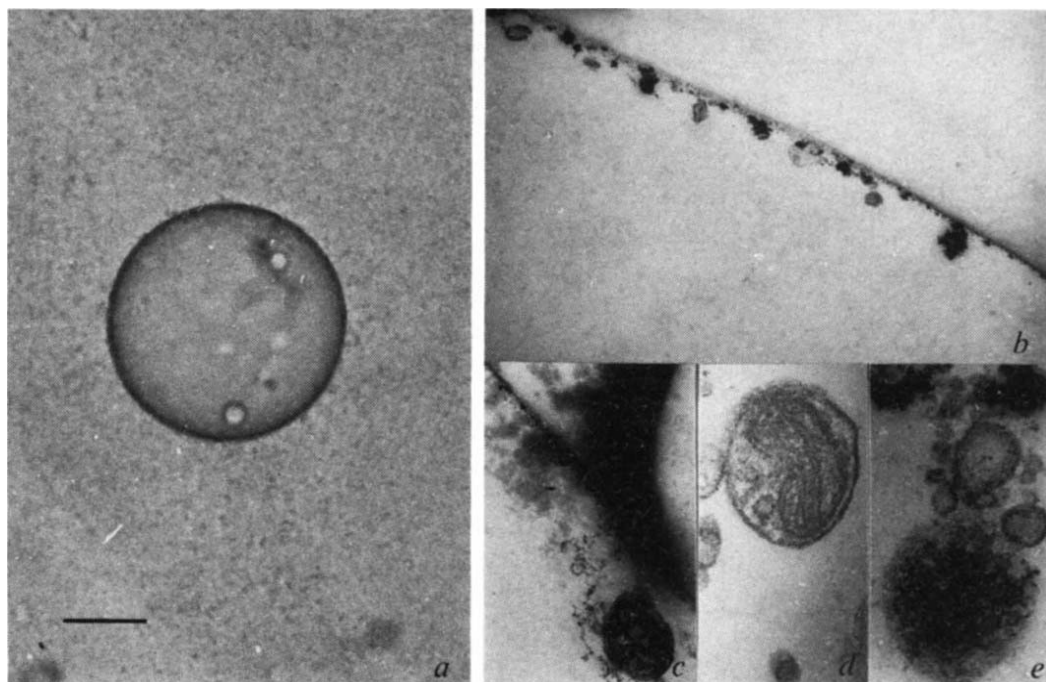
For electrophysiological investigation, the giant synaptosomes were first incubated in a solution of 190 mM potassium phosphate (pH 7.2), 10 mM NaCl, 2 mM MgCl₂ · 7H₂O and 10 mM glucose. After 10 min, the particles were transferred to physiological saline [135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 0.5 mM Na₂HPO₄, 1 mg ml⁻¹ glucose and 20 mM HEPES (pH 7.2 with NaOH)] and impaled with conventional microelectrodes (20–40 M Ω filled with 3M KCl). On electrode insertion, resting potentials in the range -45 to -76 mV (mean -55 mV; $n = 26$) were obtained (Fig. 2a). Giant synaptosomes were usually depolarized in a nernstian fashion by increasing concentrations of KCl (Fig. 2a, b). However, synaptosomes with a resting potential smaller than -50 mV often showed less than

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Fig. 1 *a*, Light micrograph of a giant synaptosome. The hazy material in the background represents unfused elements of the synaptosomal fraction. *b*, Electron micrograph showing the outer border of a giant synaptosome. Along the limiting membrane are structures which may represent small mitochondria and dense-cored vesicles. Note the lack of electron-dense organelles towards the interior of the particle. *c, d*, Examples of mitochondria found along the limiting membrane of giant synaptosomes. *e*, Cluster of vesicles, which may include synaptic vesicles, found near the surface membrane of a giant synaptosome. Scale bar corresponds to: 50 μm (*a*), 500 nm (*b, c*); 200 nm (*d*); 150 nm (*e*).

Methods: Samples were processed for electron microscopy as follows. At 4 °C:

1-h wash in physiological saline, 4–6 h of fixation in 6% glutaraldehyde in 0.1 M cacodylate buffer (*pH* always at 7.4). At 20 °C: four 1-h washes with 0.2 M cacodylate buffer, 4–8 h postfixation in 2% osmium tetroxide in 0.1 M cacodylate buffer, two 1-h washes with 0.2 M cacodylate buffer, dehydration in a graded ethanol series followed by four 1-h rinses in 100% ethanol. Samples were transferred first to propylene oxide (two 15-min exposures), then to 1:1 propylene oxide/epon (12 h) followed by evaporation of the propylene oxide and finally to epon (three 30-min changes, followed by incubation overnight at 60 °C). Throughout this procedure (except when in epon) samples were rotated slowly. Sections were cut and stained with uranyl acetate and alkaline lead citrate for viewing in a JEOL electron microscope.



nerstian responses to changes of external K^+ concentration. Equimolar replacement of either extracellular Cl^- by isethionate or Na^+ by choline had little effect on the resting membrane potential. These data suggest that K^+ permeability is the major determinant of the resting membrane potential in these structures.

Insertion of a second electrode into the giant synaptosome revealed that the interior was isopotential. When passing current through one electrode, a voltage response was recorded by the second electrode (Fig. 3). In many of the giant synaptosomes, depolarizing current pulses elicited a regenerative, transient spike which was graded with the stimulus intensity. This regenerative response was affected by changes in either external Ca^{2+} or Na^+ (Fig. 3*a, b*). The spike could be partially blocked by either Co^{2+} or tetrodotoxin (TTX) (Fig. 3*c, d*) and completely eliminated in the presence of both of these substances (Fig. 3*e*). Application of veratrine ($100 \mu\text{g ml}^{-1}$) depolarized the giant synaptosomes ($n = 3$) by 25–30 mV. Recovery of the membrane potential after veratrine required long wash-out periods (> 10 min). These data suggest that voltage-gated channels for Na^+ and Ca^{2+} are present in the plasma membrane of the fused synaptosomes. A pronounced hyperpolarization was usually

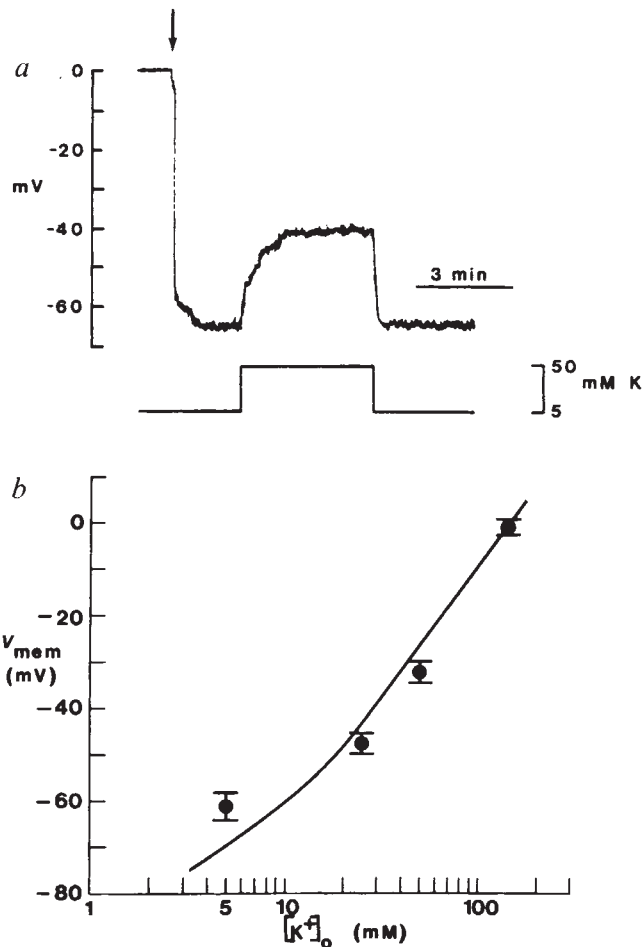


Fig. 2 *a*, Membrane potential records from a giant synaptosome. A giant particle was impaled (arrow) while in physiological saline ($\text{K}^+ = 5 \text{ mM}$). Subsequently, the K^+ concentration of the medium was increased to 50 mM (by isotonic substitution for NaCl). The membrane potential was fully recovered on removal of high K^+ . Temperature, 22 °C. *b*, Plot of membrane potential (V_{mem}) against extracellular K^+ concentration. Results are the mean $\pm$ s.e.m. of nine experiments. The curve was calculated using the equation: $V_{\text{mem}} = 2.3 (RT/F) \log([K]_o + \alpha[Na]_o) + B$, for $B = 48 \text{ mV}$ and $\alpha = 0.025$ (see ref. 8). B is equivalent to $-2.3(RT/F) \log([K]_i + [Na]_i)$, and is treated as a constant by assuming that the internal cation concentration does not change; α is equivalent to $(P_{\text{Na}}/P_{\text{K}})$, the permeability ratio between Na and K ions.

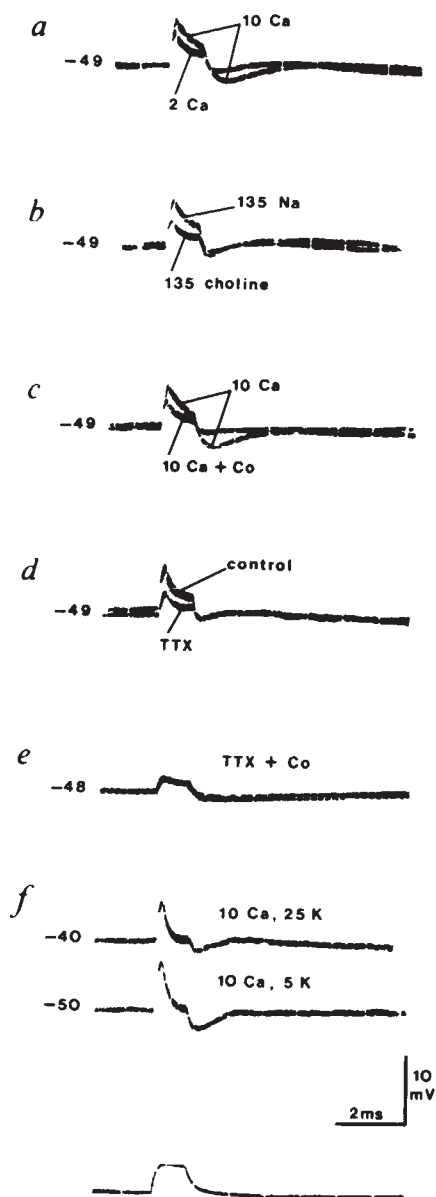


Fig. 3 Voltage records obtained during depolarizing current pulses. A single giant synaptosome was exposed to solutions of different composition as indicated: *a*, Physiological Ringer's with normal (2 mM) or high (10 mM) CaCl_2 (NaCl reduced to maintain tonicity). *b*, Physiological Ringer's with normal (135 mM) NaCl or replacement of NaCl by choline chloride. *c*, High calcium Ringer's (see *a*) or high calcium Ringer's plus 10 mM CoCl_2 (NaCl reduced to maintain tonicity). *d*, Normal Ringer's or normal Ringer's plus $1 \mu\text{g ml}^{-1}$ tetrotoxin (TTX). *e*, Normal Ringer's plus $1 \mu\text{g ml}^{-1}$ TTX and 10 mM CoCl_2 . *f*, High calcium Ringer's containing normal (5 mM) or raised (25 mM) KCl (NaCl was altered to maintain tonicity). Resting potential is indicated to the left of each trace. An example of the 100 nA current pulse used to elicit these responses is shown at the bottom of the figure. Temperature, 20°C .

observed at the offset of the stimulus (Fig. 3). This afterhyperpolarization was dependent on the concentration of extracellular calcium (Fig. 3*a*), attenuated in high K^+ solutions (Fig. 3*f*) and could be blocked by extracellular Co^{2+} (Fig. 3*c*). TTX minimally affected this hyperpolarization (Fig. 3*d*). These results are consistent with the presence of a calcium-activated K^+ conductance in these structures.

In many experiments the giant synaptosomes were sensitive to several neuroactive substances. Individually, glutamate, acetylcholine (ACh), serotonin and noradrenaline elicited depolarizing responses (Fig. 4). However, with some synaptosomes ACh induced a hyperpolarization (Fig. 4*e*). Both of these

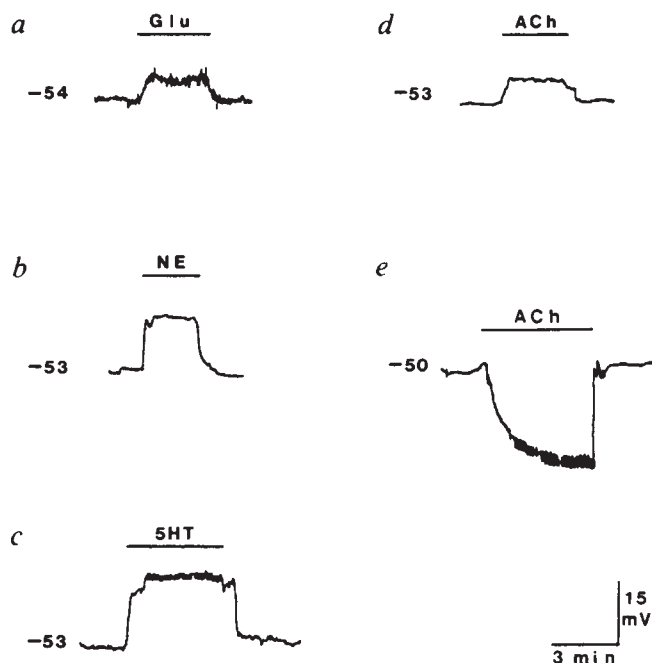


Fig. 4 Membrane response of giant synaptosomes on addition of putative neurotransmitters to the bathing medium. *a*, Glutamate (10^{-4} M); *b*, noradrenaline (10^{-3} M); *c*, serotonin (10^{-2} M); *d*, acetylcholine (10^{-2} M); and *e*, acetylcholine (10^{-2} M). Records *a* and *d* from separate giant synaptosomes; *b*, *c* and *e* from the same giant synaptosome. Resting potentials are given to the left of each trace. Temperature, 22°C .

ACh responses were reversibly blocked by equimolar atropine (data not shown). Note that the concentrations of the substances used in Fig. 4 are 10^{-4} – 10^{-2} M. However, the threshold concentration for eliciting these effects was usually 10^{-5} – 10^{-4} M.

The above results are consistent with the suggestion that the giant particles derive from the fusion of a large number of synaptosomes. These particles retain (1) mitochondria and structures resembling synaptic vesicles, (2) a stable resting potential, (3) voltage-activated Na^+ and Ca^{2+} channels and Ca^{2+} -activated K^+ channels and (4) electrical responses to putative neurotransmitter agents. These are all properties expected of presynaptic nerve terminals^{1–5}. Moreover, we failed to obtain similar structures either from crude synaptosome fractions or the other fractions from the sucrose gradient. However, we cannot exclude the possibility that both postsynaptic and non-synaptosomal elements may be involved in the formation of the particles that we obtained. Future use of antibodies that specifically recognize nerve terminals (see ref. 4) will be of value in clarifying this point.

Preparations of mammalian brain synaptosomes are heterogeneous with respect to the neurotransmitter and transmitter receptors carried by individual synaptosomes⁵. Thus, it is likely that the giant synaptosomes are obtained by the fusion of a similarly mixed population of particles. This line of reasoning suggests that studies of these particles might best be performed using populations of synaptosomes that are enriched in a particular class of nerve ending (for example, cholinergic, adrenergic). Extending this to the present data, we suggest that the high concentration of the putative neurotransmitters required to evoke the responses recorded in Fig. 4 may derive from the relative paucity of appropriate receptors on these hybrid particles. Alternatively, it may be that such presynaptic receptors do not become operational until a high concentration of the appropriate substance is present. This preparation should allow one to distinguish between these two explanations.

The preparation of these giant particles entailed the exposure of synaptosome preparations to dispase. Protease treatments have occasionally been found to encourage the fusion of other types of structures⁶. While we cannot exclude the possibility that dispase modified some of the membrane properties associ-

ated with the giant particles, we note that proteases are often used without detriment to prepare membranes for single-channel recording (see ref. 7). As such, this preparation may be used advantageously to study the characteristics of single channels and receptors in the nerve terminal membrane.

Assuming that these structures represent the fusion of large numbers of synaptosomes, one would expect a large dilution (on the basis of surface area/volume considerations) of the particle's cytoplasmic constituents by the sucrose medium in which fusion took place; this notion is consistent with the meagre amount of electron-dense material observed within these particles. However, the attendant dilution of the internal ionic milieu raises the question of how the resting potential of these particles is established. The data of Fig. 2 indicate that K^+ is the principal contributor to the resting membrane potential. Further studies should elucidate how this ion gradient is generated and maintained.

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42,000-molecular weight EGF receptor has protein kinase activity

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The epidermal growth factor (EGF) receptor and other growth factor receptors have been shown to possess tyrosine-specific protein kinase activity^{1–5}. Before the demonstration of kinase activity in growth factor receptors, tyrosine kinases of molecular weight (MW) 60,000 (60K) were found to be encoded by the *src* oncogene and other oncogenes related to *src*^{6–8}. Our earlier work on intracellular processing of the EGF receptor⁹, a 170,000-MW polypeptide, provided evidence for proteolytic separation of well defined structural domains, and suggested to us the possibility of separating functional domains by limited proteolysis. The isolation of such kinase domains should facilitate comparison of the receptor/kinase with other well characterized kinases including those of oncogene origin. We report here the identification of a catalytically functional 42K kinase derived proteolytically from the isolated human EGF receptor. This fragment, comparable in size to pp60^{src}, carries the kinase ATP-binding site, and functions catalytically even after detachment from the EGF-binding site and the major autophosphorylation region.

We used 5'-fluorosulphonylbenzoyl[¹⁴C]adenosine(5'-p- $FSO_2bz[^{14}C]A$) to screen for kinase site-containing fragments in tryptic digests of the solubilized purified receptor^{10,11}. This ATP analogue irreversibly modifies ATP-binding sites^{12–14}, and inhibits phosphorylation of both the solubilized purified receptor and a receptor-derived peptide fragment (see below).

Treatment of the freshly purified EGF receptor preparation with 50 μM 5'-p- $FSO_2bz[^{14}C]A$ at 20 °C for 30 min resulted in radiolabelling of two bands of 170K and 150K (Fig. 1a, lane a). Measurement of radioactivity in the 170K and 150K bands

revealed incorporation of 0.5–0.7 mol of 5'-p- $SO_2bz[^{14}C]A$ per mol, indicating ~1 ATP-binding site per mol of receptor molecule (measured in terms of EGF-binding sites).

Pretreatment of the receptor with 2 $\mu g ml^{-1}$ trypsin at 20 °C for 30 min, followed by incubation with 5'-p- $FSO_2bz[^{14}C]A$, resulted in the appearance of radiolabel in two bands of 150K and 42K (Fig. 1A, lane b). Pretreatment with higher concentrations of trypsin (5 $\mu g ml^{-1}$ and 9 $\mu g ml^{-1}$) resulted in covalent radiolabelling of only a single sharp band of 42K (Fig. 1A, lanes c, d). Only the 42K peptide and no other peptide smaller than 150K carried the affinity label.

The total amount of ¹⁴C-label incorporated into the three bands (170K, 150K and 42K) was the same for trypsin-treated and untreated receptor preparations. Thus, the single kinase site of the receptor polypeptide is quantitatively recovered in the 42K fragment.

Experiments were done to determine whether the pre-labelled intact receptor would generate, on trypsin treatment, a ¹⁴C-labelled 42K peptide (Fig. 1B). After labelling of the intact receptor with 5'-p- $FSO_2bz[^{14}C]A$, the unreacted reagent was removed and the preparation treated with 5 $\mu g ml^{-1}$ trypsin. Almost all the radiolabel originally present in the intact receptor band appeared in the 42K band (Fig. 1B). Thus, the receptor is the precursor of the 42K peptide, and this peptide retains the full kinase ATP-binding activity of the receptor.

The ATP analogues, AMP-PNP and 5'-p- FSO_2bzA , at a concentration of 1 mM, abolished labelling with 50 μM 5'-p- $FSO_2bz[^{14}C]A$ by more than 90% (Fig. 1C). Labelling was also abolished by pretreatment with N-ethylmaleimide or heat (Fig. 1C).

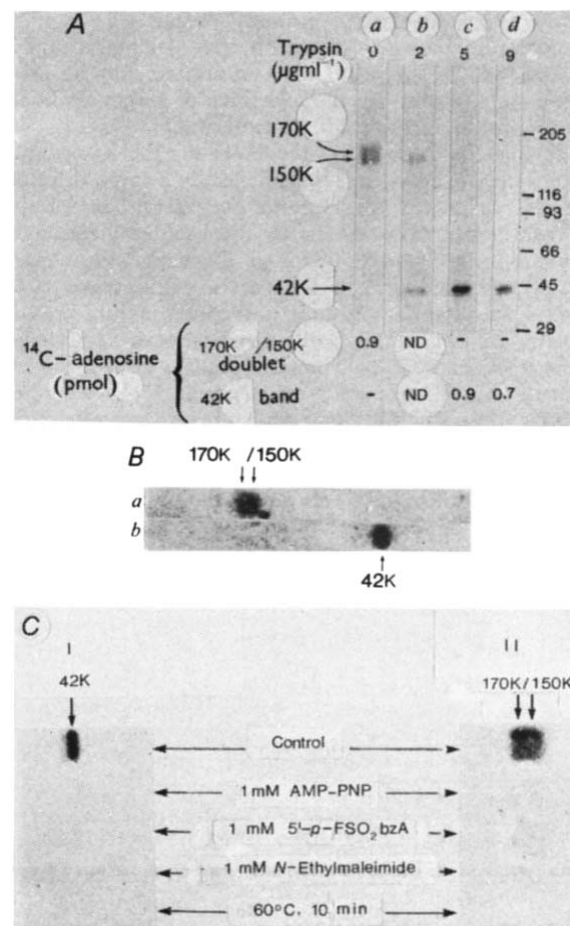
It would be interesting to know whether the 42K fragment, like other protein kinases, is capable of autophosphorylation. Phosphorylation of the purified receptor with [γ -³²P]ATP gave a major band of labelling at 170K and a minor band at 150K (Fig. 2A, lane a). When phosphorylation was preceded by a 30 min exposure of the receptor to 2 $\mu g ml^{-1}$ trypsin, the 170K band largely disappeared (Fig. 2A, lane b). Trypsin-induced conversion of the 170K form to the 150K form was associated with a large loss (>90%) of autophosphorylation sites (although, as indicated above, there was no loss of ATP-binding sites). Pretreatment of the receptor with higher concentrations of trypsin (5 and 9 $\mu g ml^{-1}$), followed by incubation with [γ -³²P]ATP, resulted in the labelling of a single sharp band of 42K (Fig. 2A, lanes c, d). Only the 42K peptide, and no other peptide smaller than 150K carried the ³²P label.

The phosphorylation that occurs on the 42K fragment as well as on the 170K/150K receptor involves tyrosine residues only (studied by standard phosphoamino acid analysis). Also, a tyrosine kinase-specific substrate, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly¹⁵, was phosphorylated by the 42K peptide at a rate of 0.7 mol peptide phosphorylated mol⁻¹ min⁻¹ (not shown). (The rate for the intact 170K receptor was 2.5 mol peptide phosphorylated mol⁻¹ min⁻¹. These results support the conclusion that the 42K peptide is an active tyrosine kinase. Several ATP analogues or pretreatment with N-ethylmaleimide or heat completely abolished the autophosphorylation of the 42K peptide (Fig. 2B). It is significant that the active sites in both the intact receptor protein and the 42K tryptic fragment exhibit similar reactivity with the reagents. The inhibitory effects of the ATP analogues could be abolished with excess [γ -³²P]ATP. The results described above indicate that the 42K fragment carries the kinase ATP-binding site and that it can be phosphorylated in the unfractionated tryptic digest.

Is the fragment a self-sufficient catalytic entity? We investigated the activity of the 42K fragment after electrophoretic fractionation of the trypsin digest on non-denaturing (SDS-free) gels (Fig. 2C). The unproteolysed receptor (170K/150K, form) was recovered largely in gel slice 2 and the 42K fragment in slice 6; these, together with the other slices, were incubated with [γ -³²P]ATP and the phosphorylated species were subsequently visualized by SDS-gel electrophoresis and autoradiography (Fig.

Fig. 1 Affinity labelling of EGF receptor fragments with a ^{14}C -ATP analogue monitored by SDS-polyacrylamide gradient gel electrophoresis. **A**, Trypsinization of the receptor, followed by incubation with 5'-p-FSO₂bz[^{14}C]A. **B**, Trypsin fragment of the pre-labelled receptor. **C**, Effect of inhibitors of EGF receptor kinase on ^{14}C affinity labelling of the 42K peptide.

Methods: **A**, Receptor (1.5 pmol, see below) was incubated at 20 °C for 30 min with the indicated amounts of trypsin (crystalline, tosylphenylethylchloromethylketone-treated; Sigma) in 20 μl of 20 mM HEPES pH 7.4, 10% glycerol, 0.2% Triton X-100. Trypsin action was stopped by adding 1 μl soybean trypsin inhibitor solution (10 mg ml⁻¹) to all tubes, followed by the addition of 5 μl of solution containing 20 mM HEPES pH 7.4, 5 mM MnCl₂. Labelling was initiated by adding 1 μl of a 1.25 mM solution of 5'-p-FSO₂bz[^{14}C]A (50 Ci mol⁻¹; NEN) in dimethylformamide. After incubation at 20 °C for 30 min, the reaction was stopped by adding rapidly in succession 2 μl of 10 mg ml⁻¹ bovine serum albumin (BSA) and 1 ml of ice-cold 10% trichloroacetic acid (TCA). The precipitate was dissolved in SDS sample buffer and subjected to 5–20% polyacrylamide gradient SDS-gel electrophoresis. After staining and destaining, the gels were prepared for fluorography by immersion in dimethyl sulphoxide (DMSO) containing 2,3'-diphenyloxazole. The gels were then autoradiographed for 40 days using Kodak X-Omat AR films and Dupont Cronex Lightening Plus intensifying screens. After autoradiography, the regions of the dried gel containing the 170K/150K doublet or the 42K band were cut out and counted using toluene-based scintillation fluid. Adjacent regions of the dried gel were similarly counted to determine background radioactivity. In these counting conditions the specific radioactivity of 5'-p-FSO₂bz[^{14}C]A was ~100 c.p.m. pmol⁻¹. **B**, Intact receptor (4 pmol) was labelled with 50 μM 5'-p-FSO₂bz[^{14}C]A as described above, then the mixture (100 μl) was diluted with 10 ml of 20 mM HEPES pH 7.4, 10% glycerol, 0.2% Triton X-100, and concentrated by Amicon ultra-filtration (using PM-30 filters) to 0.2 ml. The dilution and filtration-concentration steps were repeated three times. The ^{14}C -labelled receptor, free of excess reagent, was incubated without (**a**) or with (**b**) or 5 μg ml⁻¹ trypsin at 20 °C for 30 min. TCA-precipitable proteins were subjected to electrophoresis and autoradiography. **C**, Receptor (1.4 pmol) was either treated with 5 μg ml⁻¹ trypsin (**I**) or untreated (**II**), then incubated in the conditions indicated, followed by incubation with 50 μM 5'-p-FSO₂bz[^{14}C]A as described for **A**. Labelled proteins were isolated by acid precipitation and visualized by electrophoresis and autoradiography. Receptor isolation method: Plasma membranes were isolated from 2×10^8 human A431 epidermoid carcinoma cells as described^{10,11} using Ca²⁺ and Mg²⁺-free solutions. The membranes (2 mg protein) were stirred at 20 °C for 30 min with 1 ml of a solution containing 20 mM HEPES pH 7.4, 10% glycerol, 1% Triton X-100, and 10 μg ml⁻¹ leupeptin. After centrifugation at 100,000g for 70 min, the supernatant was added to 0.5 ml of packed EGF-agarose containing 0.4 mg of covalently bound EGF^{10,11}. After stirring at 20 °C for 30 min, the gel pellet was washed four times at 4 °C with 2 ml of a solution containing 10% glycerol and 0.2% Triton X-100, and the receptor eluted by stirring the washed gel three times (10 min at 4 °C each time) with 1 ml of 5 mM ethanolamine solution pH 10, containing 10% glycerol and 0.2% Triton X-100, and immediately brought to pH 7.0 with 0.05 M HCl¹⁰. The amount of receptor present was estimated by Scatchard analysis of ¹²⁵I-labelled EGF binding data, assuming a 1:1 complex of EGF and receptor. Recoveries varied from 40 to 60% of the input solubilized activity. The K_d binding to ¹²⁵I-labelled EGF was ~25 nM.



2B). The 42K fragment, separated in this way from the uncleaved receptor and from other receptor fragments, displayed kinase and autophosphorylating activity. These results confirm that the 42K peptide carries the entire self-sufficient kinase active site.

We investigated the relationship of the 42K fragment and the major autophosphorylation locus. Over 90% of the kinase activity sites (measured by labelling with the ^{14}C -labelled ATP analogue) in the intact receptor are recovered in the 42K peptide (Fig. 1). In contrast, only 5% of the autophosphorylation sites (measured by [γ -³²P]ATP labelling) are retained in the 42K peptide (Fig. 2A). The major autophosphorylation locus seems to lie in a 15K fragment that is cleaved off during the conversion of the 170K receptor to the 150K form (Fig. 2D). We know that the purified intact 170K receptor can maximally incorporate 6 mol of phosphate per mol of receptor. The phosphate-accepting tyrosine residue retained in the 42K peptide appears to be a minor site whose probability of phosphorylation is low, that is, the ³²P-phosphate incorporation is less than would be expected, given the presence in the peptide of one site which can be labelled. The observed low ³²P-phosphate incorporation could be due to previous occupancy of the tyrosine site with phosphate.

We next investigated the relationship of the 42K peptide to the EGF-binding site. Autophosphorylation of the 42K peptide was not stimulated by EGF (Fig. 3), and the peptide did not

bind EGF, seen by the following assays: (1) covalent attachment of ¹²⁵I-EGF^{16–18}; (2) polyethylene glycol assay¹; (3) competitive inhibition of receptor binding to EGF-agarose.

We examined whether the 42K peptide represents an adventitious protein in the receptor preparation, or pre-exists as a separable subunit of the receptor. Analysis of the receptor by sucrose density gradient centrifugation revealed the presence of only a 7.7S kinase activity peak that on labelling with [γ -³²P]ATP, showed only a 170K major/150K minor doublet band. Thus, the 42K kinase is not present in unproteolyzed receptor preparations as a separate or bound (active) entity.

Can the 42K protein pre-exist, either separately or bound to the receptor, in a catalytically inactive form that becomes active on trypsinization? This possibility is excluded by the precursor-product relationship shown in Fig. 1B. In these experiments, the ATP-binding site in the 170K receptor, covalently labelled with a ^{14}C -ATP analogue, appeared quantitatively in the 42K peptide.

These results suggest that the EGF receptor has three kinds of functional sites—the EGF-binding site, the protein kinase catalytic site and the major autophosphorylation site(s), located on separate domains. Tryptic processing of the EGF receptor results initially in the generation of an EGF-binding peptide of 150K which retains the kinase/ATP-binding site and a minor autophosphorylation site, but which has lost the major tyrosine-



Fig. 2 Phosphorylation of the 42K fragment. **A**, Trypsinization of the receptor followed by incubation of fragments with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. **B**, Effect of inhibitors of EGF-receptor kinase on autophosphorylation of the 42K peptide. **C**, Autophosphorylating activity in gel slices after non-denaturing electrophoresis. **D**, Action of trypsin on the ^{32}P -phosphorylated receptor.

Methods: **A**, Receptor (0.2 pmol) was incubated at 20 °C for 30 min with 10 μl of 20 mM HEPES pH 7.4, 10% glycerol, 0.2% Triton X-100 containing the indicated amounts of trypsin; the reaction was stopped by adding 1 μl soybean trypsin inhibitor solution (10 mg ml^{-1}) and 5 μl of a solution containing 80 mM HEPES pH 7.4 and 4 mM MnCl_2 . Phosphorylation was initiated by adding 5 μl of 60 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (70 c.p.m. fmol^{-1}). After 30 min at 20 °C, the reaction was stopped by rapid successive additions of 2 μl BSA (10 mg ml^{-1}) and 1 ml ice-cold 10% TCA. The precipitate was subjected to electrophoresis and autoradiography. **B**, Trypsin (5 $\mu\text{g ml}^{-1}$) digested (I) or undigested (II) receptor (0.1 pmol) was incubated with the indicated reagents at 20 °C for 30 min, or was heated at 60 °C for 10 min. This was followed by a 30 min incubation with 15 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Labelled proteins were isolated by acid precipitation and visualized by electrophoresis and autoradiography. **C**, Receptor (2 pmol) was incubated at 20 °C for 30 min with 80 μl of 20 mM HEPES pH 7.4, 10% glycerol, 0.2% Triton X-100 containing trypsin at different concentrations (0, 2, 5 and 9 $\mu\text{g ml}^{-1}$). After stopping the reaction with 10 μg of soybean trypsin inhibitor, the samples were subjected to non-denaturing 5–20% polyacrylamide gradient gel electrophoresis in the cold (4 °C) at pH 8.8 for 9 h (25 mA, 300–400 V). The Triton X-100-containing SDS-free gel and buffer systems used were essentially the same as those of Cohen *et al.*¹⁰. After the run, each of the four lanes was cut into 11 slices. To each slice was added 0.2 ml of a solution containing 5 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 1 mM MnCl_2 , 50 mM HEPES pH 7.4, and 10 μg of soybean trypsin inhibitor. After incubation at 4 °C for 8 h, then at 37 °C for 30 min in a shaking bath, the liquid was transferred to an Eppendorf tube, together with an additional 0.7 ml of 20 mM HEPES pH 7.4, which had been used to rinse the gel slice. Proteins were precipitated by rapid successive additions of 2 μl of 10 mg ml^{-1} BSA and 0.1 ml of 100% TCA. The precipitate was subjected to SDS-gel electrophoresis and autoradiography as described for **A**. The relatively high activity of the 42K band here may be due to its greater stability or elutability compared with the 170K/150K receptor. **D**, Intact receptor (2 pmol) was labelled with 15 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at 20 °C for 30 min, as described for **A**. The mixture (100 μl) was then diluted 200-fold with ice-cold 20 mM HEPES pH 7.4, 10% glycerol, 0.2% Triton X-100 and concentrated to 100 μl by Amicon ultrafiltration. Aliquots of the concentrate (20 μl) were incubated without (**A**) or with (**B**) 2 $\mu\text{g ml}^{-1}$ trypsin at 20 °C for 30 min, and the TCA-precipitable proteins were subjected to electrophoresis and autoradiography.

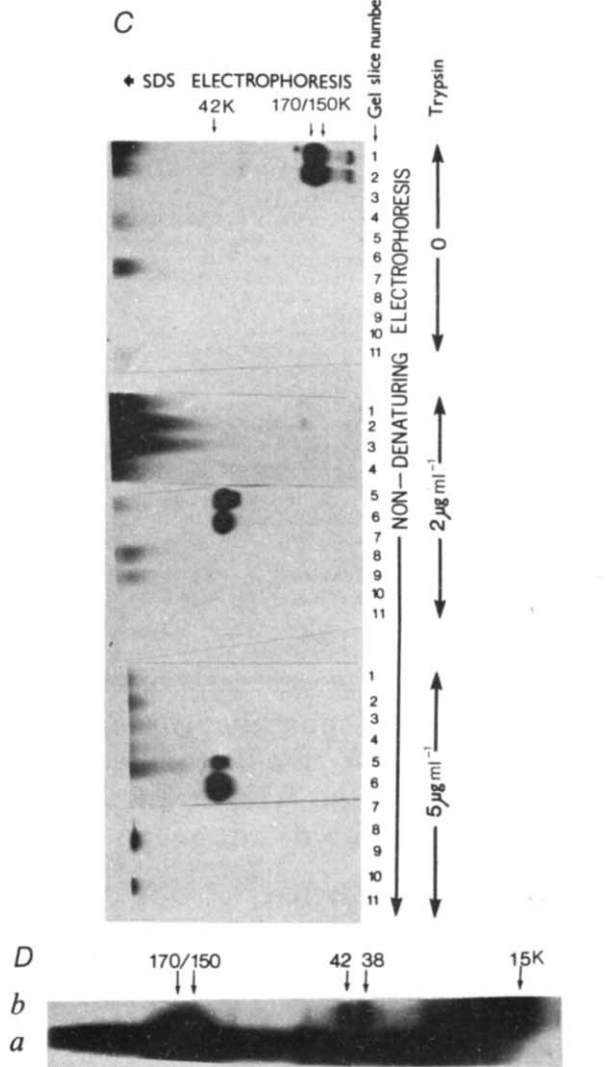


Fig. 3 Lack of effect of EGF on 42K peptide autophosphorylation. **A–F**, Crude Triton X-100 extracts of A431 membrane containing 0.1 pmol of receptor in 10 μl of 20 mM HEPES, 10% glycerol, 0.2% Triton X-100 were treated with different concentrations of trypsin at 20 °C for 30 min (lanes **A**, **B**: no trypsin; **C**, **D**: 3 $\mu\text{g ml}^{-1}$ trypsin; **E**, **F**, **E'**, **F'**: 10 $\mu\text{g ml}^{-1}$ trypsin). Action of trypsin was stopped with 1 μl of soybean trypsin inhibitor solution (10 mg ml^{-1}). The mixtures were incubated with (+) or without (–) 200 μM EGF at 20 °C for 10 min after which phosphorylation with 15 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 1 mM MnCl_2 was allowed to proceed at 20 °C for 5 min. Lanes **E'** and **F'** represent longer autoradiographic exposures for the 10 $\mu\text{g ml}^{-1}$ trypsin-treated crude extract samples. **G**, **H**, Purified receptor (0.1 pmol) treated with 5 $\mu\text{g ml}^{-1}$ trypsin was incubated at 20 °C for 10 min either with (**H**) or without (**G**) 200 μM EGF, then phosphorylated with 15 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 1 mM MnCl_2 at 20 °C for 5 min. TCA-precipitable radioactivity was subjected to electrophoresis and autoradiography.

containing phosphate acceptor domain of 15–20K. Further action of trypsin on the 150K peptide separates the kinase domain from the EGF-binding domain; a resultant 42K peptide retains both the kinase/ATP-binding site and the minor phosphate acceptor site and is catalytically functional. Note that similarly sized 30–45K phosphorylatable fragments have been described for pp60^{src} and other oncogene kinase^{19,20}. These findings and the recent demonstration of sequence homology between parts of the EGF receptor and the product of *v-erb-B*²¹, suggest that some of the tyrosine kinase oncogene products may be generated by activation of the genomic segment encoding the kinase domain of EGF receptor. Also, there exists the possibility that such tyrosine kinases may be generated by cellular processing of the EGF receptor, resulting in a loss of the control normally exerted by EGF/ligand binding.

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Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane

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The receptor for epidermal growth factor (EGF) is a 170,000–180,000 molecular weight single-chain glycoprotein of 1,186 amino acids¹. Its sequence suggests that it has an external EGF-binding domain, formed by the NH₂-terminal 621 amino acids, linked to a cytoplasmic region by a single membrane-spanning segment¹. In the cytoplasmic portion, starting 50 residues from the membrane, there is a 250-residue stretch similar to the catalytic domain of the *src* gene family of retroviral tyrosine protein kinases¹, and, indeed, a tyrosine-specific protein kinase activity intrinsic to the receptor is stimulated when EGF is bound^{2–5}. Increased tyrosine phosphorylation of cellular proteins, detected in A431 cells following EGF binding^{6,7}, may be important in the mitogenic signal pathway. Tumour promoters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), counteract this increase^{8,9}, as well as causing loss of a high affinity class of EGF binding sites⁹. The major receptor for TPA has been identified as the serine/threonine-specific Ca²⁺/phospholipid-dependent diacylglycerol-activated

protein kinase, protein kinase C^{10–14}. By substituting for diacylglycerol, TPA stimulates protein kinase C. Protein kinase C phosphorylates purified EGF receptor at specific sites, and this reduces EGF-stimulated tyrosine protein kinase activity⁸. TPA treatment of A431 cells increases serine and threonine phosphorylation of the EGF receptor at the same sites^{8,15}, which suggests that the reduction of EGF receptor kinase activity in TPA-treated cells is a consequence of the receptor's phosphorylation by the kinase. We have attempted to identify these phosphorylation sites and show here that protein kinase C phosphorylates threonine 654 in the human EGF receptor. This threonine is in a very basic sequence nine residues from the cytoplasmic face of the plasma membrane in the region before the protein kinase domain; it is thus in a position to modulate signalling between this internal domain and the external EGF-binding domain.

Although treatment of A431 cells with TPA causes increased phosphorylation of the receptor on both threonine and serine residues, the only change detectable by tryptic peptide mapping is the appearance of three new phosphothreonine (PThr)-containing phosphopeptides, peptides X, Y and Z (Fig. 1), which are also the sites phosphorylated by protein kinase C *in vitro*⁸. We therefore concentrated on identifying these sites of threonine phosphorylation in the EGF receptor. Because the *v-erb-B* gene product is very similar to part of the EGF receptor¹⁶, and reasoning that the phosphorylation sites would lie in the cytoplasmic region of the protein, we scanned the predicted *v-erb-B* protein sequence¹⁷ for likely sites of threonine phosphorylation. Many protein kinases select their target amino acids through recognition of the primary sequence neighbouring the hydroxyamino acid, with positively or negatively charged amino acids being the key determinants in such sequences. Although it is not known whether protein kinase C is similar in this regard, it will phosphorylate many basic proteins *in vitro* such as histone H1, high mobility group (HMG) proteins and myelin basic protein^{18–21}. We therefore focused on threonines with highly charged environments. Thr 98 of the predicted *v-erb-B* protein is contained in the sequence Arg-Arg-Arg-His-Ile-Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg (Fig. 2); if it were phosphorylated, the limit tryptic digest of this sequence would yield the peptide PThr-Leu-Arg. Our experiments on separations of peptides on cellulose thin layers by electrophoresis and chromatography suggest that the mobility of this peptide would be very similar to that of peptide X in the EGF receptor. Peptide X is not susceptible to digestion by *Staphylococcus aureus* V8 protease (cleavage at Glu), *Pseudomonas fragi* protease (cleavage at Asp and cysteic acid) or chymotrypsin (cleavage at Phe, Tyr and Trp). We therefore synthesized the peptide PThr-Leu-Arg (Fig. 1). This peptide co-migrates precisely with peptide X in our standard two-dimensional separation conditions (Fig. 1); the complete predicted sequence of the human EGF receptor¹ shows that the sequence surrounding Thr 654 is identical to that in the equivalent region of the *v-erb-B* protein (Fig. 2). Because residues 654–656 are the only place in the EGF receptor from which Thr-Leu-Arg can be generated by trypsinolysis¹, peptide X must correspond to residues 654–656.

We next investigated peptides Y and Z. We suspected that they might be related to peptide X, as they were not susceptible to digestion by *S. aureus* V8 or *P. fragi* proteases or chymotrypsin, and their two-dimensional mobilities could be accounted for by the addition to peptide X of one and two extra basic residues respectively. The sequence embedding Thr 654 is likely to give rise to products with additional basic residues at either terminus of the limit digest peptide due to the inability of trypsin to act as an exopeptidase. To test whether all three peptides indeed form a nested set, we synthesized a 24 amino acid peptide corresponding to residues 87–110 of the predicted *v-erb-B* protein (this is identical to the sequence of the EGF receptor between residues 643 and 666 except for the first two residues, and contains one threonine and no serines). This peptide is phosphorylated *in vitro* by a purified preparation of protein kinase C (Fig. 3a). The only detectable phosphoamino acid in the resultant labelled phosphopeptide is PThr (Fig. 3b). Two-

Fig. 1 Tryptic phosphopeptides of A431 cell EGF receptor and synthetic *v-erb-B* peptide. *a*, 700 Cerenkov c.p.m. tryptic digest of ^{32}P -labelled EGF receptor from untreated A431 cells (72 h). *b*, 900 Cerenkov c.p.m. tryptic digest of ^{32}P -labelled EGF receptor from TPA-treated A431 cells together with 1 μg PThr-Leu-Arg (72 h). *c*, 1,000 Cerenkov c.p.m. tryptic digest of ^{32}P -labelled phosphorylated synthetic peptide with 1 μg PThr-Leu-Arg (24 h). *d*, 900 Cerenkov c.p.m. tryptic digest of ^{32}P -labelled EGF receptor from TPA-treated A431 cells plus 400 Cerenkov c.p.m. tryptic digest of ^{32}P -labelled phosphorylated synthetic peptide (72 h). The positions of peptides X, Y and Z previously shown to be phosphorylated by protein kinase C are indicated. PThr-Leu-Arg, where present, was detected by staining with ninhydrin. Its position is given by a diagonal arrow on panel *c*, while in panel *b* it migrates with the spot arrowed X. **Methods:** Two subconfluent 5-cm dishes of A431 cells were labelled with 1 mCi ^{32}P -orthophosphate for 16 h, then one culture was treated with 100 ng ml $^{-1}$ TPA dissolved in dimethyl sulphoxide for 60 min and the other with an equivalent amount of dimethyl sulphoxide before isolation of the EGF receptor by immunoprecipitation as described previously⁸. Tryptic peptides were prepared from ^{32}P -labelled EGF receptor as outlined previously³⁰. The peptide Tyr-Leu-Arg-Arg-Arg-His-Ile-Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-Leu-Gln-Glu-Arg-Glu-Leu-Val-Glu-NH₂ was synthesized using a Beckman 990 synthesizer and purified by ion exchange and partition chromatography. The peptide was homogeneous on HPLC analysis and had the expected amino acid composition. This peptide was phosphorylated by protein kinase C as follows. Peptide at a final concentration of 0.2 mM was incubated at 30 °C for 10 min in 20 μl of a buffer containing 20 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.5 mM CaCl₂, 5 mM dithiothreitol, 60 μg ml $^{-1}$ phosphatidylserine and 6 μg ml $^{-1}$ diolefin together with 10 μCi [γ - ^{32}P]ATP (3,000 Ci mmol $^{-1}$, Amersham) and 0.05 units kinase C purified from rat brain by a modification of the procedure of Kikkawa *et al.*³¹ including DE52 cellulose chromatography, Ultragel ACA 34 filtration and hydroxyapatite chromatography steps. The protein kinase C preparation had a specific activity of ~ 150 U mg $^{-1}$ at 22 °C using histone H1 as substrate. The phosphorylated peptide was isolated by electrophoresis for 35 min at 1 kV on a 100- μm cellulose thin layer plate. 3×10^5 Cerenkov c.p.m. of peptide were recovered following elution and 10^4 c.p.m. digested with 2 μg trypsin in 10 μl 0.025 M NH₄HCO₃ for 2 h at 37 °C. The peptide PThr-Leu-Arg was synthesized as follows. tBOC-phosphothreonine was prepared by treatment of 100 μmoles D,L-phosphothreonine (Sigma) with 200 μmoles tBOC-azide (Aldrich) in 500 μl water/dimethylformamide/pyridine (25:25:1) for 24 h at 23 °C. The tBOC-PThr was recovered by stepwise elution from DE52 cellulose with ammonium acetate pH 4.5. tBOC-PThr, which eluted at 0.5 M, was desalted following lyophilization by gel filtration on Sephadex G-15 in water (yield of free acid 10%). 6 μmoles tBOC-PThr were coupled to 6 μmoles Leu-Arg (Bachem) in water (pH adjusted to 5.0) with 10 μmoles 1-ethyl-3-(3-dimethylamino propyl)carbodiimide (Calbiochem-Behring) for 16 h at 23 °C. Following deblocking with trifluoroacetic acid for 30 min at 23 °C, the major product was separated from PThr and Leu-Arg by electrophoresis at pH 4.7 for 30 min at 1 kV on a 100- μm cellulose thin layer plate (yield 5%). This peptide contained phosphate and had the expected amino acid composition. The resultant samples were analysed on a 100- μm cellulose thin layer plate by electrophoresis at pH 8.9 for 20 min at 1 kV (horizontal dimension, with a centre origin (arrowhead) and anode to the left) and chromatography (vertical dimension)³⁰. Exposure times are measured with an intensifying screen at -70 °C.

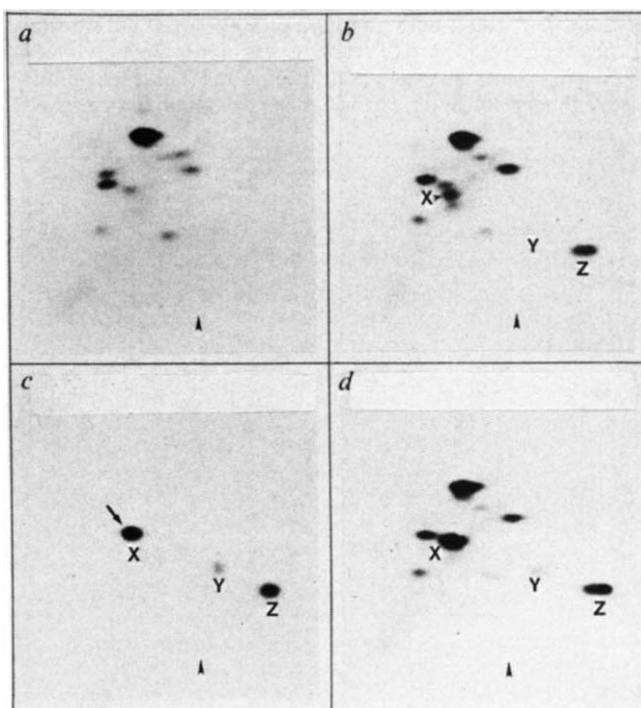
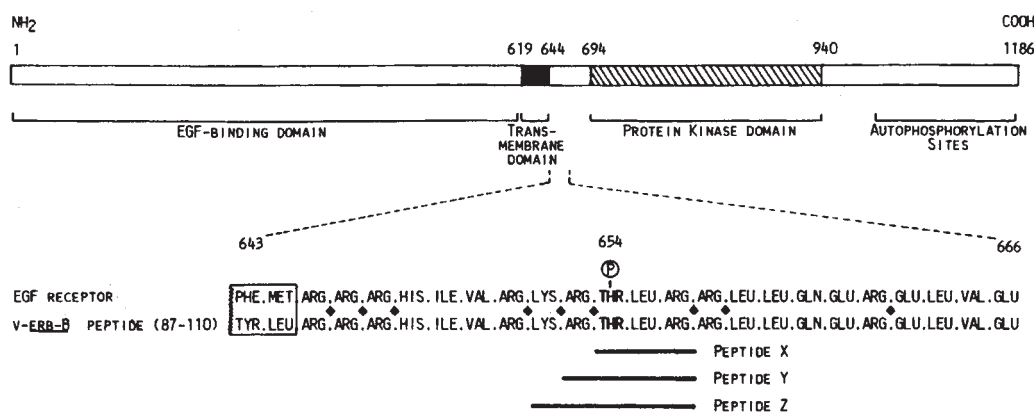


Fig. 2 Schematic diagram showing the site of protein kinase C phosphorylation in the EGF receptor. The receptor is depicted as a bar, with amino acid numbers given above taken from the sequence of Ullrich *et al.*¹. The proposed transmembrane domain is filled, while the protein kinase domain is hatched. The sequence of the human EGF receptor from residues 643 to 666 is shown with the homologous sequence from the *v-erb-B* gene product aligned below^{1,17}. The *v-erb-B* sequence shown is that of the synthetic peptide used in Figs 1 and 3. The two residues at the cytoplasmic end of the proposed transmembrane domain are boxed at the left-hand side of these sequences. Note that the EGF receptor and *v-erb-B* sequences differ only in these two residues. Potential sites of trypsin cleavage are marked as diamonds between the two sequences. The heavy bars below the sequences indicate the deduced structures of peptides X, Y and Z.



dimensional separation of a tryptic digest of this phosphopeptide shows three peptides with mobilities very similar to peptides X, Y and Z, which co-migrate with those peptides on analysis of a suitable mixture (Fig. 1).

The precise structures of peptides Y and Z were determined by Edman degradation of ^{32}P -labelled peptides obtained from a digest of the phosphorylated synthetic peptide. After each cycle, a fraction of the sample was analysed by electrophoresis, without removal of the cleaved NH₂-terminal residue. All the

radioactivity is released from peptide X at cycle 1 as expected (Fig. 3c), but the majority of the radioactivity is not released from peptide Y until cycle 2 (not shown). Sequential cycles convert peptide Z to peptide Y and then to peptide X (Fig. 3c). Prolonged digestion of peptide Z with a vast excess of trypsin also converts it inefficiently into peptide X (not shown). We therefore deduce that the structures of peptides Y and Z are Arg-PThr-Leu-Arg and Lys-Arg-PThr-Leu-Arg respectively.

The phosphorylation of the synthetic *v-erb-B* peptide by pro-

tein kinase C requires both Ca^{2+} and phospholipid (not shown). The K_m for peptide in this reaction was approximately $5 \mu\text{M}$ and the $V_{\max}$ $5 \mu\text{mol per min per mg}$ (Fig. 3a). This high affinity suggests that protein kinase C recognizes predominantly primary

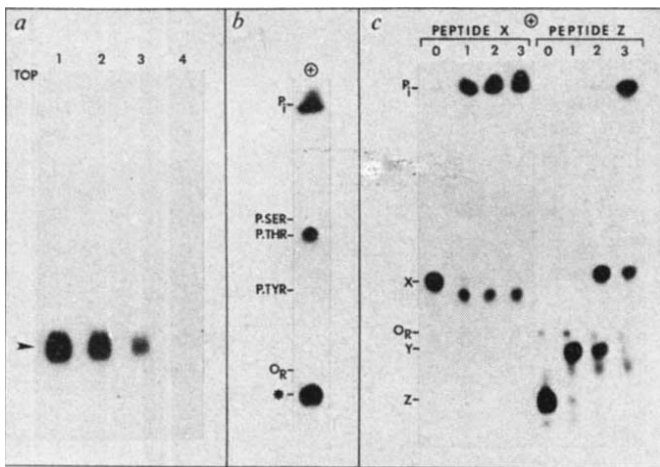


Fig. 3 Phosphorylation of synthetic peptide. **a**, Synthetic peptide at the following concentrations was phosphorylated as described in Fig. 1 legend except that the final concentration of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was $10 \mu\text{M}$ (specific activity $10 \mu\text{Ci nmol}^{-1}$) and the incubation time at 30°C was 4 min: lane 1, $3.0 \mu\text{M}$; lane 2, $1.5 \mu\text{M}$; lane 3, $0.75 \mu\text{M}$; lane 4, no peptide. The reactions were stopped by the addition of EGTA to 2 mM and freezing. One μl of each sample was analysed by SDS-polyacrylamide electrophoresis on a gel containing 25% acrylamide, 0.05% bisacrylamide. The dried gel was exposed to X-ray film for 2 h. The arrowhead indicates the position of the phosphorylated peptide which co-migrated with marker glucagon (3,500 molecular weight). The band in lane 1 contained 1.7×10^3 Cerenkov c.p.m. **b**, ^{32}P -labelled synthetic peptide (1,500 Cerenkov c.p.m.) was hydrolysed in 6 M HCl for 1 h at 110°C and then analysed by electrophoresis at pH 3.5 (origin indicated O_R , anode at top) on a 100- μm cellulose thin layer plate for 30 min at 1 kV with internal phosphoamino acid standards. The plate was exposed to X-ray film for 12 h at -70°C with an intensifying screen. The positions of the marker phosphoserine (P.SER), phosphothreonine (P.THR) and phosphotyrosine (P.TYR) detected by staining with ninhydrin are given. Also indicated are the positions of orthophosphate (P_i) and a phosphopeptide (*) which was identified as PThr-Leu by co-migration with synthetic PThr-Leu. **c**, Purified ^{32}P -labelled peptides X and Z (7,000 and 13,000 Cerenkov c.p.m. respectively) were taken through three cycles of Edman degradation as follows. Peptides were dissolved in 40 μl pyridine/water (50:50) containing 5% phenylisothiocyanate and incubated at 45°C for 30 min. The reactions were then extracted twice with 200 μl heptane/ethyl acetate (10:1) and twice with 200 μl heptane/ethyl acetate (2:1). The organic phases were discarded and the aqueous phases were lyophilized. The residues were taken up in 50 μl trifluoroacetic acid and incubated at 45°C for 10 min to cleave the NH_2 -terminal amino acid from the peptide. The trifluoroacetic acid was removed *in vacuo* and the residues were dissolved in 20 μl H_2O . Five μl were sampled and the remainder was taken through two further cycles in identical fashion with the equivalent of one-quarter of the original material being sampled at each stage. One-third of the samples from each cycle and an equivalent amount of untreated peptide were analysed by electrophoresis at pH 8.9 (origin indicated O_R , anode at top) for 20 min at 1 kV on a 100- μm cellulose thin layer plate. The plate was exposed to X-ray film for 12 h at -70°C with an intensifying screen. The positions of peptides X, Y and Z and orthophosphate (P_i) are indicated. Lane 0, starting material; lane 1, sample after first cycle; lane 2, sample after second cycle; lane 3, sample after third cycle. The acid-catalysed cyclization reaction of a phenylisothiocyanate-reacted peptide possessing an NH_2 -terminal phosphothreonine or phosphoserine leads to the elimination of much of the phosphate during the cleavage rather than the formation of an anilinothiazolinone derivative. The minor spot migrating just below the position of peptide X may be anilinothiazolinone phosphothreonine. Note that the analysis of this type of experiment will be more complicated if the peptide contains a group reactive with phenylisothiocyanate in addition to the $\alpha\text{-NH}_2$ group.

sequence determinants in its substrates. The sequence in the EGF receptor is the first described for a site phosphorylated by protein kinase C *in vivo*. It will be possible to produce synthetic variants of this sequence to identify residues which are important for recognition by the kinase. In myelin basic protein, the site has features similar to that in the EGF receptor, with basic residues at positions two and eight to the NH_2 -terminal side of the phosphorylated Ser 115 (ref. 22). A synthetic peptide based on this sequence serves as a good substrate for protein kinase C (R. S. Turner, J. F. Kuo and B. E. Kemp, personal communication). Similarly, Ser 24 phosphorylated in HMG 17 is preceded by basic residues at positions 1, 2, 7 and 9 (ref. 21), and Ser 35 phosphorylated in histone H1 by basic residues at positions 2, 3, 4, 8 and 9 (ref. 18). Thus, protein kinase C appears to belong to the growing number of protein kinases which favour basic residues in the vicinity of their substrate hydroxyamino acid. These include the cyclic AMP- and cyclic GMP-dependent protein kinases, myosin light-chain kinase, phosphorylase kinase and the multi-functional calmodulin-dependent protein kinase.

Very little is known about physiological substrates for protein kinase C. TPA-induced serotonin release from platelets correlates well with protein kinase C-mediated phosphorylation of a 40,000 molecular weight protein²³. The EGF receptor, however, is the first example where phosphorylation by protein kinase C of a purified protein has been shown to have a functional consequence. The normal role of the protein kinase C-mediated phosphorylation of the receptor may be to act as a feedback mechanism to attenuate the response to EGF either by blocking kinase activation or by decreasing EGF binding affinity⁹. The feedback would be modulated via diacylglycerol whose concentration rises as a result of EGF-induced turnover of phosphatidylinositol²⁴. This is in agreement with the increased phosphorylation of the receptor at the protein kinase C-specific sites in EGF-treated A431 cells¹⁵.

We have shown here that there is a single major threonine phosphorylation site for protein kinase C in the EGF receptor at position 654. Our previous measurements indicate that up to 35% of receptor molecules are phosphorylated at this threonine in response to either phorbol ester or indole alkaloid tumour promoters⁸. The active form of kinase C is largely membrane associated, where it is bound to phospholipids and either its natural activator diacylglycerol or a substitute activator such as TPA²⁵. The position of Thr 654 spaced nine amino acids from the cytoplasmic side of the proposed membrane-spanning segment puts it in an ideal position to be phosphorylated by protein kinase C. The 50 residues between the membrane and the start of recognizable homology with the tyrosine protein kinases may act as a 'linker' between the EGF-binding domain and the protein kinase domain, and as such might be involved in signal transmission between the two upon EGF binding.

It is not known how the binding of EGF increases the activity of the receptor tyrosine protein kinase. There could be a direct allosteric conformational change induced by EGF binding, although it is unclear how this might be transmitted across the membrane. Alternatively EGF-dependent clustering of the receptor may be required for activation. Because the basic residues in the 'linker' are likely to interact with the phosphate head groups of plasma membrane phospholipids, the introduction of a phosphate group in this region with its potential as an alternative charge acceptor could well have significant effects on both tyrosine protein kinase activity and EGF binding, whatever mechanism is involved. Our results suggest that alteration of Thr 654 or neighbouring residues by site-directed mutagenesis and the use of antibodies recognizing this region may further our understanding of signal transduction.

As TPA markedly reduces EGF binding to many mitogenically responsive cell types²⁶⁻²⁹, it is of interest to determine whether protein kinase C mediates threonine phosphorylation of the EGF receptor in such cell types in a manner similar to A431 cells. An investigation of whether *v-erb-B* proteins are phosphorylated at the equivalent site also seems warranted.

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Autophosphorylation sites on the epidermal growth factor receptor

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The epidermal growth factor (EGF) receptor is a tyrosine-specific protein kinase with autophosphorylating activity¹⁻⁴. A 300 amino acid-long region of the receptor's cytoplasmic domain matches (35-90 % homology) sequences of transforming proteins from the *src* family⁵ and includes a putative nucleotide binding site⁶. Several of the *src* transforming proteins have tyrosine kinase activity⁷, but *v-erb-B*, which appears to be a truncated EGF receptor, is virtually identical to the receptor over this region and yet lacks detectable kinase activity^{8,9}. To locate possible acceptor sites in the *v-erb-B* protein, we have mapped these sites in the human EGF receptor. We report here that three tyrosine sites near the C-terminus are phosphorylated *in vitro*. In intact cells, we find that EGF stimulates phosphorylation of several sites, the tyrosine 14 residues from the C-terminus being modified the most extensively. The equivalent site is absent in the *v-erb-B* protein of avian erythroblastosis virus (AEV) and may influence tyrosine kinase activity^{10,11}.

Knowledge of the complete primary structure of the human HEGF receptor⁵ is used here to map the tyrosine acceptor sites for the receptor kinase autophosphorylation activity. We purified EGF receptors from detergent lysates of A431 cells using R1 monoclonal immunoaffinity chromatography and performed *in vitro* autophosphorylation by addition of [γ -³²P]ATP to immobilized receptor while in an immune complex¹² in the presence or absence of EGF (Fig. 1). After tryptic digestion of the receptor, peptides were resolved by reverse-phase HPLC. We found that phosphate-labelled peptides (P1, P2 and P3; Fig. 1a) eluted at three distinct acetonitrile concentrations as reported previously¹³. The most hydrophobic peptide, P3, was partially resolved into two components, a and b. Thin-layer electrophoretic analysis of acid hydrolysates¹⁴ from either receptor or purified peptides demonstrated that tyrosine was the only amino acid phosphorylated *in vitro* in the conditions used. Only

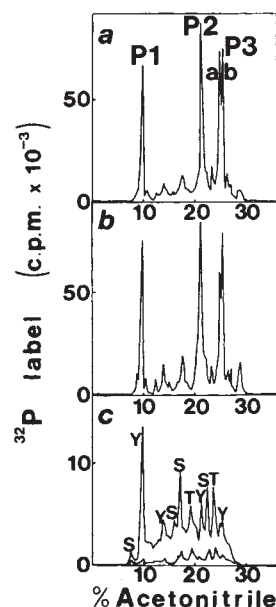


Fig. 1 Tryptic phosphopeptide maps of EGF receptor from A431 cells. **a**, EGF receptor was purified from A431 cells as described previously¹². Autophosphorylation was performed with 150 μ g receptor as washed immunocomplex with monoclonal antibody R1-Sepharose. The receptor was incubated for 1 h at 20 °C in 1 μ g ml⁻¹ EGF, 150 mM NaCl, 1 mM MnCl₂, 50 mM HEPES pH 7.4, 0.1 % Triton X-100. [γ -³²P]ATP was then added (20 μ M, specific activity 690 c.p.m. pmol⁻¹) for 10 min. Reagents were then removed by filtration and the receptor washed, eluted, fully reduced and alkylated, further purified by gel permeation chromatography, digested with trypsin and run on reverse-phase HPLC at pH 2.0, all as described previously¹². Fractions (0.3 ml) were collected at a flow rate of 1.5 ml min⁻¹ and a gradient of 1 % acetonitrile min⁻¹. The ³²P-phosphate label in each fraction was determined by counting Cerenkov radiation and is plotted against the percentage acetonitrile in the fraction. **b**, Same as **a** except that autophosphorylation was performed in the absence of EGF (replaced by 1 μ g ml⁻¹ bovine serum albumin, BSA). The length of the incubation in the presence of [γ -³²P]ATP was increased to 60 min. **c**, Two subconfluent 15-cm dishes of A431 cells were labelled for 14 h, each with 5 mCi ³²P-orthophosphate in Eagle's medium containing 5 % normal phosphate concentration and 0.5 % fetal calf serum. Upper line, 1 μ g ml⁻¹ EGF was added to one dish for 10 min before lysis in 100 mM HEPES pH 8.5, 50 mM NaF, 1 mg ml⁻¹ BSA, 1 mM Na₃VO₄, 10 mM NaP_i pH 8.5, 5 mM *p*-nitrophenylphosphate, 100 mM NaCl, 1 mM EGTA, 1 % Triton X-100, 10 mM benzamide, 50 μ g ml⁻¹ phenylmethylsulphonyl fluoride, 10 μ g ml⁻¹ soybean trypsin inhibitor. Kinase activities were inhibited by chelation of divalent metal ions. The extract was clarified by centrifugation, then 50 μ g of R1-Sepharose added for 90 min at 4 °C. The immobilized antibody was washed thoroughly and receptor eluted as described¹². The *in vivo* labelled receptor was then mixed with 150 μ g of unlabelled purified EGF receptor as a carrier and processed as described in **a**. The phosphoamino acid content of each peak was determined by partial acid hydrolysis and thin-layer electrophoresis¹⁴; the predominant phosphoamino acid is given above each peak. Lower line, the second dish was processed as above except the cells were not treated with EGF before lysis (replaced by 1 μ g ml⁻¹ BSA). Both dishes had the same cell density before labelling. The amount of receptor immunoprecipitated was the same from each plate as determined by Coomassie blue staining of SDS-polyacrylamide gels.

the rate of phosphorylation seemed to be altered by EGF, because identical peptides were phosphorylated in its presence or absence over longer incubation times (Fig. 1b).

To characterize the kinase autophosphorylation sites used *in vivo*, we labelled A431 cells with ³²P-phosphate (Fig. 1). We fractionated tryptic phosphopeptides by HPLC and determined the phosphorylated amino acids by thin-layer electrophoresis¹⁴. Ten distinct phosphopeptides were found, four containing phosphotyrosine, four phosphoserine and two phosphothreonine. In previous reports, fewer phosphopeptides were detected¹⁵. Three

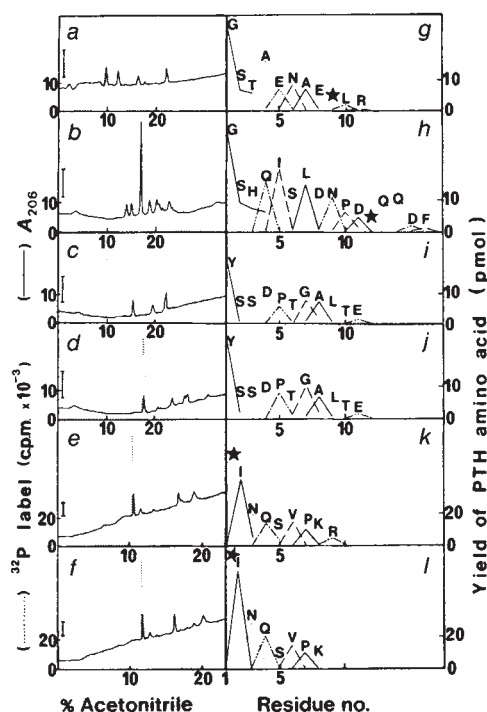


Fig. 2 Purification and sequencing of phosphopeptides. **a**, The fractions labelled P1 in Fig. 1a were pooled and applied to a Synchropak RPC 18 reverse-phase HPLC column (4.6 × 75 mm; Synchrom, Linden, Indiana) run in 10 mM ammonium acetate buffer at pH 6.5 with a linear gradient of acetonitrile of 1 % min⁻¹ at 1.5 ml min⁻¹. Fractions (0.3 ml) were collected and counted for Cerenkov radiation. The absorbance of the eluate at 206 nm was monitored (the bar represents 0.01 absorbance units). 310 pmol of labelled phosphate were loaded onto the column. **b**, The fractions HHlabelled P2 in Fig. 1a were run as described above for **a**; 360 pmol were loaded. **c**, Fractions labelled P3a in Fig. 1a were run as in **a**; 220 pmol were loaded. **d**, Fractions labelled P3b in Fig. 1a were run as in **a**; 270 pmol were loaded. **e**, The fractions corresponding to P3a from a second identical preparation of *in vitro* labelled EGF receptor were digested with *Staphylococcus aureus* V8 protease (1 µg in 10 µl in 50 mM ammonium bicarbonate, pH 7.5 for 24 h at 37 °C). The resulting mixture of peptides was separated by reverse-phase HPLC at pH 2.0 (ref. 12). Fractions (0.3 ml) were collected. The linear gradient was 0.5% acetonitrile min⁻¹. The bar represents 0.01 absorbance units. **f**, The fractions corresponding to P3b were digested and separated as in **e**. **g**, The fractions containing the peak of phosphate label from **a** were pooled; the 70 pmol of phosphate label present were divided into two halves which were sequenced on gas phase sequencers^{21,22}. The yield of PTH amino acid (identified above the peak by the one-letter code) is plotted against cycle number of the Edman degradation. **h**, Sequence of the phosphopeptide from **b**; 45 pmol of phosphate label were loaded for each sequencer run. **i**, Sequence of phosphopeptide from **c**; 25 pmol phosphate label loaded. **j**, Sequence of phosphopeptide from **d**; 30 pmol phosphate label loaded. **k**, Sequence of phosphopeptide from **e**; 85 pmol phosphate label loaded. **l**, Sequence of phosphopeptide from **f**; 105 pmol phosphate label loaded. * Phosphotyrosine residues.

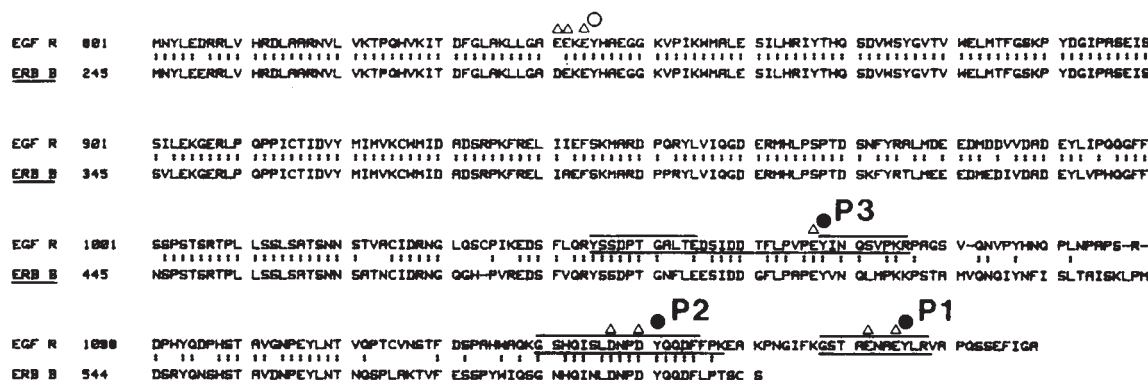


Fig. 3 The alignment of the phosphate acceptor regions of the EGF receptor with the *v-erb-B* protein sequence. (Sequence of the EGF receptor from ref. 5, sequence of the *v-erb-B* protein from AEV-H, from ref. 10.) The tyrosine residue at 845 (○) in the EGF receptor sequence is homologous to the pp60^{v-src} phosphoacceptor site¹⁹. ●, EGF receptor phosphorylation sites P1, P2 and P3; △, acidic residues at phosphorylation sites. The peptide sequences determined in Fig. 2 are overlined, the predicted complete sequence of each tryptic phosphopeptide is underlined. The last four residues of the *v-erb-B* protein are derived from the viral *env* gene¹⁰.

of the four phosphotyrosine peptides co-chromatograph at two different pHs with peptides P1, P2 and P3 (data not shown). Addition of EGF to intact cells caused a general increase (5-fold) in the extent of receptor phosphorylation with a marked selective increase (10-fold) in the extent of labelling of peptide P1 (Fig. 1c). This is a considerably larger stimulation than that reported previously, which may be due to the use of protease inhibitors that prevented loss of sites near the C-terminus.

To locate the phosphorylation sites, pmol amounts of phosphate-labelled peptides were purified¹² and sequenced^{16,17} (Fig. 2). In each case we located our established sequence in the amino acid sequence of the EGF receptor (Fig. 3). At the ninth degradation cycle of P1, we recovered no phenylthiohydantoin (PTH) amino acid although the cDNA sequence predicted a tyrosine residue. In gas phase sequencing, phosphotyrosine PTH cannot be detected¹⁸. Because we knew that the phosphate in this peptide occurs in phosphotyrosine and that the peptide only contains one tyrosine residue, the phosphate could be assigned unequivocally to residue nine. Similarly, we assigned residue 12 as phosphotyrosine in P2. Peptides P3a and P3b gave the same sequence up to residue 11; however, none of the residues

assigned was a phosphotyrosine that was presumably carboxy-terminal to this sequence. We digested peptides P3a and P3b with *Staphylococcus aureus* V8 protease and purified (Fig. 2e, f) and sequenced (Fig. 2k, l) the resulting phosphopeptides. P3a had an additional C-terminal arginine residue compared with P3b and the N-termini could be assigned as phosphotyrosine.

Thus the major *in vitro* autophosphorylation sites of the EGF receptor are assigned as follows: P3, tyrosine 1,068; P2, tyrosine 1,148; and P1, tyrosine 1,173. Each of these residues is immediately preceded by an amino acid residue with an acidic side chain. These sequences are similar to most of the tyrosine phosphorylation sites described previously^{19,20}. Tyrosine 1,173 is the site selectively phosphorylated *in vivo* while tyrosines 1,148 and 1,068 are autophosphorylation sites used to a lesser extent *in vivo*. A third minor *in vivo* tyrosine autophosphorylated peptide has not yet been located. The major autophosphorylation site that is selectively phosphorylated *in vivo* (P1) is 14 residues from the carboxy-terminus of the EGF receptor and is not found in the *v-erb-B* protein from the *erb-B* gene of AEV (AEV-H) which is fused at the carboxy-terminus to four residues

of the viral *env* protein (Fig. 3)¹⁰. Therefore, *v-erb-B* is probably terminated prematurely with respect to the cellular sequences from which it is derived. P1 has the same neighbouring V8 protease and trypsin cleavage sites as predicted for the major *in vivo* autophosphorylation site of the EGF receptor²¹. The autophosphorylation sites P1, P2 and P3 used *in vitro* do not correspond to the tyrosine phosphorylated in one member of the *src* family, pp60^{v-src} (ref. 19), which would be tyrosine 845 in the EGF receptor. These sites are not shared with any other members of the *src* family of oncogene products except *v-erb-B*.

The absence of the site homologous to P1 in the *v-erb-B* protein may contribute to the failure to detect kinase autophosphorylation activity in AEV-transformed cells. C-terminal truncation can influence tumorigenicity since the deletion mutant AEV-H, td-130, which has a large C-terminal truncation, will induce sarcomas but not erythroblastosis in chickens¹⁰. On the other hand, avian leukosis virus can induce erythroblastosis by insertion of a viral long terminal repeat into *c-erb-B* with consequent N-terminal truncation but presumably without affecting the C-terminus of the protein product²². As yet the significance of EGF receptor tyrosine autophosphorylation is unclear despite work performed on two other members of the *src* family, pp60^{v-src} (ref. 23) and p130^{gag-fps} (ref. 24); it remains to be seen if either or both amino- and carboxy-terminal truncations of the receptor are essential for generation of a transforming signal.

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(*Hin*) form) and comprises ~10% of the population⁴. Excision of *Tc1* from its chromosomal location in the Bergerac strain is indicated by the presence, on genomic blots, of a minor band corresponding to the size of the uninserted restriction fragment^{1,5,6}. Here we describe the recovery of extrachromosomal linear and closed circular copies of *Tc1* from the Bergerac strain, presumably a result of *Tc1* excision.

We initially looked for a *Tc1* transcription product using Northern blots containing RNA isolated from two strains of *C. elegans*. RNA preparations from the Bergerac strain contained *Tc1*-hybridizing sequences (Fig. 1). Attempts to demonstrate that the *Tc1*-hybridizing material was RNA revealed that this material was resistant to alkali hydrolysis and ribonuclease digestion, but sensitive to restriction enzyme digestion. These results indicated that extrachromosomal copies of *Tc1* had co-precipitated with RNA in the extraction conditions used here⁷. The *Tc1*-hybridizing material was observed in the Bergerac strain, but not in the Bristol strain, although the methods used would probably not have detected the 10-fold lower levels that would be expected in the Bristol strain. Some *Tc1*s from the Bristol strain have been cloned and found to have restriction maps identical to those isolated from the Bergerac strain, indicating that they are potentially functional elements (N. Mawji, unpublished results). The presence of extrachromosomal *Tc1* is not limited to the Bergerac strain; it has also been detected in other wild-type and laboratory strains of *C. elegans* (data not shown).

To investigate the *Tc1*-hybridizing material further, DNA was isolated by CsCl-ethidium bromide ultracentrifugation from the Bergerac strain. Individual fractions were collected from the gradient, electrophoresed on agarose gels, blotted onto nitrocellulose, and tested for the presence of *Tc1*-hybridizing material (Fig. 2). A large amount of *Tc1*-hybridizing material is present in the fractions corresponding to the genomic DNA. This material presumably represents the 300 or so copies of *Tc1* that are integrated throughout the genome. There is also a band in the region of the gradient which corresponds to linear copies of extrachromosomal *Tc1* (fractions 3, 4). Below the main genomic band on the gradient, a minor band containing *Tc1*-hybridizing material was detected in fractions 5 and 6. Restriction digestion was used to demonstrate that the material in fractions 5 and 6 is in fact closed circular *Tc1*.

The *Tc1*-hybridizing material in fractions 5 and 6 from the CsCl gradient, corresponding to a linear fragment of 950 base pairs (bp), was purified on an agarose gel and analysed by gel electrophoresis. In the undigested sample, two bands of *Tc1*-hybridizing material were apparent (Fig. 3, lane 1). The major band has an apparent size of 950 bp, consistent with its being a closed circular molecule the size of *Tc1* (1,610 bp). The minor band presumably consists of nicked circles that were generated after isolation of the closed circular material from the gradient fractions. When digested with *ClaI*, an enzyme that has a single cleavage site in *Tc1*, a single band of ~1,600 bp is observed (data not shown). When digested with *XhoI*, a band of ~1,400 bp is observed (Fig. 3, lane 2). *XhoI* digests *Tc1* at two

Isolation of the closed circular form of the transposable element *Tc1* in *Caenorhabditis elegans*

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The mobilization of *Tc1*, a transposable element in the genome of the roundworm *Caenorhabditis elegans*, has been investigated. Genomic blot hybridization has shown that *Tc1* exists in very different numbers in the genomes of two closely related strains of *C. elegans*^{1,2}; there are ~30 copies of *Tc1* in the Bristol strain, whereas in the Bergerac strain there are 200–300. Most of these *Tc1* elements are structurally highly conserved^{1–3} although there exists a second form which contains a *HindIII* restriction site (*Tc1*

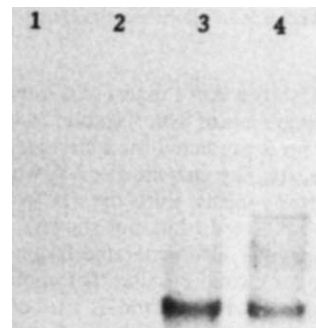


Fig. 1 Autoradiograph of *Tc1*-hybridizing material detected in RNA preparations: lanes 1, 2, Bristol strain; lanes 3, 4, Bergerac strain.

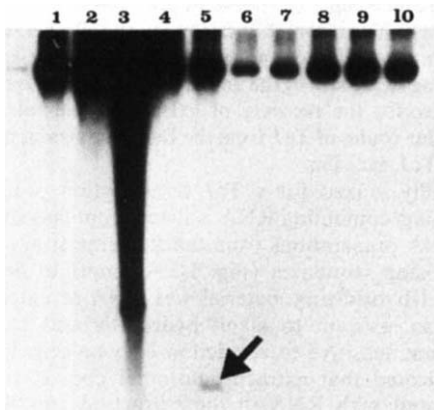


Fig. 2 Isolation of extrachromosomal closed circular and linear copies of *TcI*. Bergerac DNA was prepared¹ and centrifuged on a CsCl-ethidium bromide equilibrium gradient⁵. Fractions were collected and the DNA recovered and electrophoresed on a 0.8% agarose gel. DNA was transferred to nitrocellulose⁹ and hybridized with ³²P-labelled *TcI* (*Hin*) DNA. Filters were hybridized overnight and autoradiographed for 5 days using Kodak Blue Brand film. Lane numbers correspond to CsCl gradient fraction numbers. The arrow indicates the closed circular *TcI*.

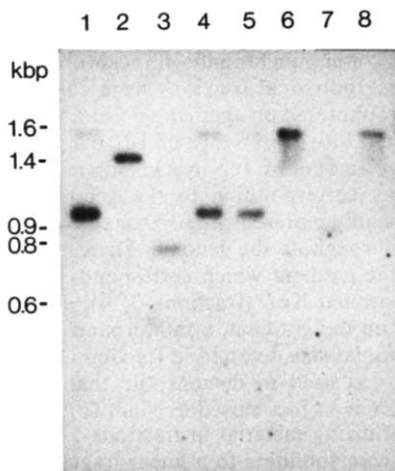


Fig. 3 Autoradiograph of restriction digests of the extrachromosomal *TcI*-hybridizing material. Before digestion, the extrachromosomal *TcI* from the CsCl fractions was further purified to remove the high-level contamination of chromosomal *TcI* seen in Fig. 2. Agarose gel fractions corresponding to sizes of either 1,610 bp (linear) or 950 bp (closed circular) were electroeluted and electrophoresed in a 0.8% agarose gel. Lane 1, undigested material from CsCl fractions 5 and 6. Lanes 2-5, restriction digests of this material: *XhoI* (lane 2); *HinfI* (lane 3); *HindIII* (lane 4); *EcoRI* (lane 5). Lane 6, undigested material from CsCl fraction 3. Lanes 7, 8, restriction digests of this material with *XhoI* and *EcoRI*, respectively. The sizes of the fragments are indicated at the left-hand side in kilobase pairs (kbp).

sites, 220 bp apart, thus a *XhoI* digest of a linear *TcI* molecule would give three fragments of 890, 500 and 220 bp. The observed fragment of 1,400 bp is predicted for a circular molecule closed at the inverted repeats. The enzyme *EcoRV*, which cuts once in each of these inverted repeats, shifts the size from 950 bp to just under 1,600 bp as predicted (data not shown). Digestions with other restriction enzymes also generated fragments of the sizes predicted assuming a closed circular *TcI* molecule was being digested (Fig. 3). No *EcoRI* or *HindIII* sites could be detected in the extrachromosomal material (lanes 4 and 5), as expected from the published restriction maps of *TcI* (refs 1, 3). If the *TcI* (*Hin*) form⁴ was present in proportion to its chromosomal

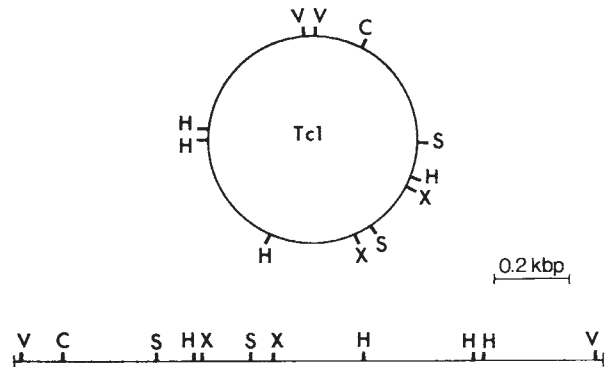


Fig. 4 Restriction map of closed circular and linear *TcI*: V, *EcoRV*; X, *XhoI*; H, *HinfI*; S, *SalI*. *TcI* lacks *EcoRI* and *HindIII* sites.

representation, ~10% of the molecules would be expected to linearize with *HindIII*.

Lane 6 of Fig. 3 contains material of 1,600 bp from fraction 3 of the CsCl gradient that had been purified on an agarose gel, and corresponds to linear copies of extrachromosomal *TcI*. Restriction digestion of this material with *XhoI* gave a 890-bp fragment (lane 7); a fragment of this size is predicted if the DNA contains linear molecules of *TcI* with unique ends at the inverted repeats (see above and restriction maps of linear and closed circular *TcI* shown in Fig. 4). The large number of linear molecules detected is intriguing, but their significance to *TcI* transposition is unclear.

We have demonstrated the recovery of extrachromosomal copies of *TcI* from the Bergerac strain of *C. elegans*. High-frequency excision of *TcI* from somatic tissue has been demonstrated previously⁶ and is presumably the source of the extrachromosomal linear and closed circular *TcI*s demonstrated here. At present we have no evidence linking the events of excision and closing of linear *TcI*s to heritable events, such as the mutations that result from reintegration of *TcI* into germ-line chromosomes. Indeed, high-frequency excision has been shown to be limited to somatic tissue⁶. The role of either the linear or closed circular molecules in the transposition process in *C. elegans* and other organisms is unknown. Extrachromosomal copies of the *copia* family of transposable elements in *Drosophila melanogaster* have been described⁸, but their role in transposition is unknown. *Copia* transposable elements have direct terminal repeats, in contrast to *TcI* elements which have inverted repeats; thus, the conditions and mechanisms by which these two transposable elements circularize are presumably different. *Copia* elements can be mobilized by hybrid dysgenic crosses, but it is unknown whether the closed circular form is involved. *TcI*, on the other hand, appears to be constitutively excising in the Bergerac strain. The generation of new mutations resulting from excision or integration of *TcI* is being investigated in our laboratories.

We thank Dr D. L. Baillie for valuable discussion and financial support and N. Mawji and K. Beckenbach for technical assistance. This research was supported by grants from the NCI (Canada) to A.M.R. and D.L.B.; the MRC (Canada) to A.M.R.; NSERC to Dr. D. L. Baillie; and an NSERC scholarship to T.P.S.

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Sex and drugs . . . and science

John Treherne

Strong Medicine. By Arthur Hailey.
Michael Joseph with Souvenir Press/Doubleday: 1984.
Pp. 430. £9.95, \$16.95.

From the writer who brought to millions the glitter of *Hotel*, the drama of *Airport*, the intrigue of *The Moneychangers*, this immensely powerful novel takes the reader deep into the heart of the pharmaceutical industry where millions of dollars and millions of lives hang on every decision ... and into the heart and mind of an unforgettable woman.

THE manifest enthusiasm of Mr Hailey's publishers for his latest production even extends to sending a review copy to *Nature*. Evidently they hope to reach the hearts and minds of the scientific community, as well as selling the film rights. Their optimism is not entirely misplaced. There is widespread public and professional concern about the activities of international pharmaceutical companies; readers of the 9 August issue of this journal will recall Carl Djerassi's angry review of Gary Gereffi's book on the drugs industry and the Third World, under the headline "Making drugs (and soaking the poor?)"

There might be doubts about the depth of Mr Hailey's fictional study, but there can be none about the swiftness with which he grapples with his subject. In the Prologue we are "up forward in first class" on a Boeing 747 amidst caviare and champagne — next year (i.e. 1985). Eight lines down the first page, Celia de Grey, the international drugs tycoon, reminds her handsome, supportive husband, Dr (of medicine) Andrew Jordan, that *certain deaths may be drug-related*. Two pages later we are back in New Jersey in 1957. The harassed young Dr J. (attending the critically ill wife of a factory hand in a thunderstorm), gets very cross with Celia, the beautiful saleswoman from Felding-Roth Pharmaceuticals, who obligingly returns to the hospital with an untested drug called Letromycin. She rivets the doctor with blazing eyes and persuades him to administer Letromycin — and saves his patient. After this Celia never looks back: she is gratefully embraced by and, within another page, married to Dr J. ("a quiet civil wedding with a few close friends and relatives"); she persuades Felding-Roth *not* to market Thalidomide, has a baby, flings her weight about at sales conferences, has another baby and by 1963 is on the fast track at Felding-Roth.

The break-neck passage of time is signalled by the regular insertion of tense paragraphs seemingly culled from *Keating's Contemporary Archives*. In 1972 ("The rock-music cult called 'Woodstock Nation' flared briefly, then burned out") Celia is despatched to Britain to nose out

any local pharmacological talent. Between shopping trips to Harrods, she finds her way along Tennis Court Road to "Cambridge University's Biochemistry Building", there to seek the brilliant young Martin Peat-Smith who is in close pursuit of a memory-enhancing brain peptide, which, he hopes, will "perhaps eliminate mental ageing altogether". Celia is appalled by the gloomy squalor of what seems to be embarrassingly like a contemporary Cambridge research laboratory, is hardly mollified to be told that Fred Sanger once worked upstairs, but is much taken by Peat-Smith who is swiftly translated to the luxurious Felding-Roth laboratory at Harlow. There he gets to work to isolate Peptide 7, sacks the tight-lipped sadistic animal technician Gertrude Tilwick, seduces her beautiful replacement, Yvonne, and, eventually, Celia herself. There are quite a lot of spicy bits: Peptide 7 is not only an elixir but, it turns out, a powerful aphrodisiac *and*, apparently, a slimming agent.

Not surprisingly, Peptide 7 is an instant success; long lines form at pharmacy counters and Felding-Roth is well on the way to an increase in revenue of six hundred million dollars. Peat-Smith is elected to the Royal Society and there are rumours of a knighthood and a Nobel Prize. Meanwhile, Felding-Roth achieve another coup with Hexin W (a drug of rather vague pharmacological properties), the brainchild of the unpleasant Dr Vincent Lord who was very put out by the success of Peptide 7.

Unfortunately, Hexin W is discovered to have nasty side-effects and poor Celia (now the boss of Felding-Roth) is soon in trouble with the Federal Drug Administration and the persistent and unscrupulous Senator Donahue, who is intent on her destruction. Nevertheless, she manages a brief trans-Atlantic dash, next year (i.e. 1985), to witness Peat-Smith's investiture at Buckingham Palace. Yvonne (Lady Peat-Smith), looking lovelier than ever thanks to Peptide 7, is naturally very disappointed at not meeting Princess Diana, but excited by the prospect of Sir Martin's New-Blood post at Cambridge.

And that is where we came in, 400 pages before, with poor Celia returning to America to face the music. Will the Felding-Roth code of practice stand up to scrutiny by a Senate investigative committee?

Arthur Hailey's latest offering will doubtless help to relieve the tedium of

many an uncritical hour up forward with caviare and back in economy class with a hamburger. Despite the cardboard characters and a strong-bronzed-arms-and-yielding-breasts approach, *Strong Medicine* does make the attempt to write about important issues in a surprisingly fair and even-handed fashion; for most readers it will probably constitute their only extensive reading on this matter of vital public concern. So we should, indeed, be grateful to the creator of Celia de Grey, Gertrude Tilwick, Peptide 7 and the nubile Lady Peat-Smith. □

John Treherne is University Reader and Honorary Director of the Agriculture and Food Research Council's Unit in the Department of Zoology, University of Cambridge. He is author of several novels, the most recent of which, The Trap, will be published early next year by Jonathan Cape.

Making scents

C.T. David

Chemical Ecology of Insects.
Edited by W.J. Bell and R.T. Cardé.
Chapman & Hall/Methuen: 1984.
Pp. 524. £30.

THE subject of chemical ecology embraces everything of ecological significance that is mediated in any way by olfaction or taste. It thus encompasses a vast amount of information and a great range of different behaviours. A full account of the area would be expected to include material about pheromones, about chemical influences on interactions with other organisms — the finding of plant and animal hosts by smell, for example, and in prey detection and predator avoidance — and also about reactions to other chemicals in the environment.

It would seem that any book which attempted to cover the effects of all of these semiochemicals would be superficial; but *Chemical Ecology of Insects* proves that need not be the case. The editors have provided us with a selection of detailed and interesting chapters — starting with mechanisms of chemoreception; leading on through odour dispersal, insect orientation mechanisms to distant odour sources, plant-host finding, plant-herbivore relations, predators and parasites, chemical protection (including a long chapter on mimicry which seems more to do with "visual" than with chemical ecology), and spacing and aggregation; and ending with chapters on the pheromones of bark beetles and social insects. The only serious omission is of a discussion of the relations of insects to vertebrates.

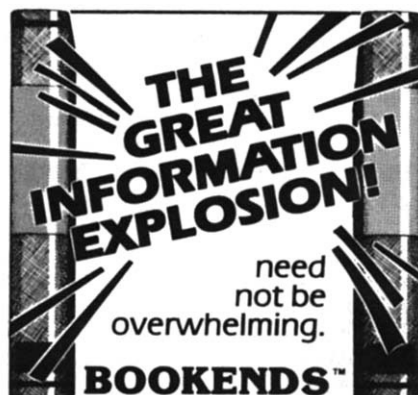
Such a range of material cannot be dealt with in detail here. So I will concentrate, quite invidiously, on one of the many good

chapters in the book, and on the only bad feature.

Almost every other book covering insect pheromones discusses the maximum distance of effective pheromone communication on the basis of estimates of the time-averaged pheromone concentration at different distances downwind from the emitter, using three assumptions — that insects have a constant threshold for response to pheromone; that the response time of the insects is long compared to the time of passage past them of the turbulently dispersed puffs of pheromone; and that the wind direction is constant. All of these assumptions are wrong. In their chapter on odour dispersal, Elkington and Cardé give an excellent appraisal of the problem. They discuss the limits of time-average models, put forward other models, and assemble much experimental evidence to produce better ways of estimating the area from which insects might be attracted to an odour source.

The bad feature is the index. There are 324 entries only, quite inadequate for a book of this length. Publishers should not need to be told that ancillary features such as this make all the difference between a book being read and it being left untouched in the library. □

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Political resources

Stephen Cotgrove

Development and the Environmental Crisis: Red or Green Alternatives?

By Michael Redclift.

Methuen: 1984. Pp. 149. Hbk £9.50, pbk £4.25.

WHAT is the connection between feminism, ecology and Marxism? And how, in association, might they save the environment of the underdeveloped world? It is these questions that Michael Redclift sets out to examine.

Marxists, however, have little to say about man's relations with nature — in general they see environmental problems as the outcome of the predatory and exploitative character of capitalism, and nature as an inexhaustible resource. So one wonders why Redclift spends so much of his short book exposing the inadequacies of Marxism. And while it is true that radical thinking and the emergence of new political parties has brought together an uneasy coalition of feminists, ecologists, anarchists and liberals in a search of an alternative to market-dominated, ecologically damaging and consumer-orientated industrialization, this book lacks an analysis of the constituencies from which such movements are drawn and their potential as bases from which political challenges may grow. It is a pity that the political dimension of environmental protection is explored within a somewhat partisan selection of political ideas.

Nevertheless we do need reminding that the environmental crisis is a global one and cannot be divorced from the concept-

ualization and analysis of development and the problems of the South. In his discussion of technology and the control of resources (Chapter 6), Redclift argues strongly that technologies are not politically neutral, either in their origins or consequences. The "Green Revolution" did not simply increase crop yields. It also redistributed income and wealth, materially affected land tenure institutions, introduced new marketing arrangements . . . and disrupted the ecology of natural resources [p.107].

Wealthy farmers who have access to credit and are better able to buy fertilizers and machinery flourish, and the gap between large and small farmers widens. By contrast, research in farming systems is intended to benefit those who employ their own resources and to increase yields without the same social consequences. Again, water technology

provides opportunities for entrepreneurship which have important distributive consequences . . . In much of South Asia the control over water exercised by bureaucracies has enabled them to wield considerable political power [p.31].

The central lesson of *Development and the Environmental Crisis* is that protection of the remaining forests, and improvement in crop yields and in irrigation, are not simply technical questions which can be solved by conservation strategies. Both development and environmental protection are inextricably tied up with vested interests and the political and institutional arrangements which they support. But this book is unfortunately short on strategies which offer hope, and its style and organization tend to obscure the importance of the message. □

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Questions of the Quaternary

D. Q. Bowen

Late-Quaternary Environments of the United States. Vol. 1 The Late Pleistocene, edited by Stephen C. Porter. Vol. 2 **The Holocene**, edited by H.E. Wright Jr.

University of Minnesota Press/Longman: 1984. Vol. 1 pp. 407, \$45, £45. Vol. 2 pp. 277, \$45, £45.

THESE two volumes arise from a series of meetings between American and Russian scientists, at Moscow and Baku (1976), New York and Salt Lake City (1978), and Irkutsk and Yakutsk (1980), sponsored by a co-operative agreement in environmental protection. Volume 1 covers the period 25,000 to 10,000 years ago, Vol. 2 10,000 years ago to the present. Both are subdivided into sections with specialist chapters.

To a large extent environments in the United States were climatically controlled by the Laurentide ice-sheet, not least during its decay. H.E. Wright Jr shows how the persisting ice, until 6,500 years ago in Labrador, may have caused increased seasonality in climate beyond its borders. Herein may be the explanation for the disharmonious faunas and floras of the late-glacial and early Holocene. Such increased seasonality and a picture of the time-transgressive response of natural systems to the forcing effect of climatic change (whereby the United States was transformed from a full glacial into a full interglacial state), form the background to the complex set of questions examined in the books. Here readers will be grateful to the editors for their helpful introductions.

Resolution of the issues, however, relies on adequate evidence and good-quality dating control. Almost inevitably, in a terrain as large and diverse as the United States, both will be uneven. Thus there is a contrast between the middle west — with its impressive isopol maps showing the development of Holocene vegetation

(described here by Webb *et al.*) — and the west where detailed work on well-dated sites (Baker) is still required. The deficiency is remedied in part by the successful studies on packrat (*Neotoma*) middens (Spaulding *et al.*), though this work too needs to be extended beyond the south-west.

In a similar vein, the advance of montane ice is less well documented than that of the continental ice-sheet: in general the onset and decline of montane glaciation runs ahead of the continental ice-sheet, no doubt because of the lagged response of the latter to climatic change.

Of further importance in establishing time-transgressive events for use in climatic modelling are data showing falling pluvial lake levels some 13,000 years ago in the southern Basin and Range, but not until the end of the Pleistocene farther north (Smith and Street-Perrot). It is difficult to disagree with R.G. Barry in his call for the collection of even more accurately dated data as a prelude to realistic modelling of the contemporary atmosphere.

All will welcome the first major synthetic treatise on the Holocene — from the latest assembly of palaeoecological data on vegetational history (discussed, for example, by Heusser), at first colonizing, later in dynamic equilibrium and subsequently coping with a cooling which culminated in renewed montane glaciation (Burke and Birkland); to models of alluvial history reconciling vegetation and climate (Knox); to the pragmatic and careful evaluation of Kutzbach on climatic change; and to a review of archaeology (Aikens).

These two volumes follow in the tradition of *The Quaternary of the United States* (Princeton University Press, 1965). But times have changed. The books focus on the past 25,000 years only, a necessity arising from the vast array of data available, but also because of the relatively good dating controls available for that period. The restriction is also in part a response to the high level of sophistication now demanded of Quaternary data. This places the earlier Wisconsinan (last glaciation) and Last Interglacial in a somewhat depressing light, to say nothing of the Quaternary *terra incognita* before that time. Perhaps it will be one of the many triumphs of the present volumes that, as well as catalysing further work on the late Quaternary, they may focus some attention on the seemingly intractable problems of that earlier time — without the solution of such problems, the absence of a full explanation of the dynamics of atmospheric-oceanic-continental interaction through time will continue to frustrate full understanding of even the late Quaternary and the projection of its climatic trends to the future. Happily these new volumes will provide a visionary nudge forward along these highways. □

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Alternative forms in bioenergetics

Hans V. Westerhoff

Physics of Bioenergetic Processes.

By L.A. Blumenfeld.

Springer-Verlag: 1984. Pp. 132. DM 70, \$27.20.

RATHER than writing a much-needed handbook on our knowledge of physics as applied to bioenergetics, Professor Blumenfeld claims that enzymic catalysis cannot be satisfactorily accounted for by the current principles of physical chemistry. He rejects the relevance of one of those principles — the Eyring equation — for enzyme reactions, as it assumes equilibrium between the transition state complex and substrate-plus-enzyme. Experimental evidence is reviewed indicating that shortly after undergoing a chemical transition from E to E^{*}, the conformation of an enzyme may still correspond to the equilibrium conformation of state E. Because this conformation generally does not correspond to the most stable conformation for state E^{*}, the enzyme will relax to a new conformation, E^{*}. The transition state, says Blumenfeld, thus cannot be described as having an enzyme in a conformation defined by its chemical state. As an alternative, he proposes to describe conformational relaxation in mechanical terms.

In the usual — and quite successful — approach, the two states E^{*} and E are just included as separate states in the des-

cription. It is characteristic of the book as a whole that Blumenfeld does not explain why such a treatment would fail.

The author also calculates that, for a gas engine of molecular dimensions, the information-energy needed to move the piston at zero rate would exceed the amount of work done. Thus molecular machines could not operate reversibly. Here, the flaw in the argument lies in the strictly mechanical interpretation of reversibility; reversibility merely implies that the ensemble (time) average of the piston movement is zero. Individual pistons may fluctuate rapidly. A similar snag is evident in Blumenfeld's alternative theory for energy transduction, which at the level of a single particle in a quantum mechanical box uses Newton's action = -reaction law.

The review sections in the book provide new information only where they describe results previously published in Russian. That information is useful, but it is clear that the *internal* inconsistencies in the chemiosmotic coupling theory suggested by Blumenfeld exist only in his interpretations of that theory. With respect to external inconsistencies, it is not news that the chemiosmotic theory does not survive all experimental tests.

Although slow conformational changes may prove to have a role in bioenergy transduction, Blumenfeld's assertion that already established physical chemical theories could not account for them is unconvincing. His alternative theory is even less so. □

Hans V. Westerhoff is a visiting scientist at the National Institutes of Health, Bethesda, Maryland.

Selected theory

Mark Ridley

Conceptual Issues in Evolutionary Biology: An Anthology.

Edited by Elliott Sober.

MIT Press: 1984. Pp. 725. Hbk \$40, £38; pbk \$19.95, £18.95.

WHAT is our modern philosophy of evolution? To judge from the generous, and, I think, representative selection of previously published works in Elliott Sober's anthology, it is mainly concerned with the "designful" properties of organisms — their adaptations — and with Darwin's explanation of adaptation, natural selection.

The most damaging philosophical criticism of Darwin's theory is that it does not explain adaptation at all. Natural selection, in Spencer's form, "the survival of the fittest", has often been said to be a circular argument; but all the relevant authors in this anthology would disagree. The point is here most clearly made by Gould. "Fitness" has two meanings: the number of offspring on average left by a type, and

how well designed, in the engineer's sense, the type is. The fitness of an eye can be considered optically as well as reproductively, and in Darwin's theory the latter is the cause (but not the criterion) of the former. If anything, I found five papers spread through 80 pages too large a dinner, but this is the one part of a subject, often criticized for its interminable and sterile controversies, on which all the professionals now agree. Let us indulge the celebration.

If natural selection does explain adaptation, we can proceed to three more questions. For the benefit of what kind of entities does natural selection produce adaptations? How well adapted are those entities? What kind of explanation is it? Now the disagreement begins. The first question is professionally phrased as "What is the unit of selection?". The main answers on offer are the gene, the organism and the group; and the main controversy concerns Williams and Dawkins's argument in favour of the gene. Fifteen pages of Dawkins (no Williams) stand against 95 from Gould, Lewontin, Sober and Wimsatt who variously argue for selection at "higher levels"; but unfortunately the

main arguments for Williams and Dawkins's position are nowhere explained. The Dawkins paper (on the "extended phenotype") aims to extend, rather than defend, his case, and his critics, although they describe (sometimes even fairly) the theory they oppose, do not pause to expound it.

The argument, in condensed form, is this. The unit of selection will be, in a sense, the unit of heritability, because natural selection cannot act on non-heritable entities. Organisms are not inherited, because they die; "higher genetic levels" are not inherited, because they are broken and recombined: the unit of heritability will be something smaller, which Williams and Dawkins have chosen to call (and *define* as) the gene. If, in the kind of complex situations that Wimsatt, Sober and Lewontin discuss, that unit is longer than a cistron, then the gene (in Williams and Dawkins's sense) is longer than a cistron too.

In practice, however, Williams and Dawkins do not apply their argument to the problems of population genetics, but use it to think about what kind of adaptations will be favoured in particular environments. That application is strictly circumscribed. Williams and Dawkins have not been trying to explain everything in biology solely in terms of genes. Wimsatt, however, directs his criticism against a hopelessly ambitious theory of genic selection, which he has practically pumped up into *ex DNA omnia*:

T2: (Dynamical thesis): Processes at the genetic level determine (and are the primary and ultimate) explanations for processes at all higher levels.

It is quite easy to make mincemeat of this, especially if "genetic level" is identified with a single genetic locus. Wimsatt only needs to argue "that understanding evolutionary processes requires the invocation of causal mechanisms and nomic regularities" concerning things other than genes, for his work of destruction, against T2, to succeed. Against Williams and Dawkins, however, he is rather wide of the mark.

Actually, because genes are propagated in sets by the thousand through organismic agency, the selection of genes often produces organismic adaptations. There is broad agreement that organismal (rather than genic or group) adaptation is common, the disagreement being mainly about whether organismal adaptation is caused by the selection of genes or "higher levels". But either way we can ask how important the theory of natural selection is by asking how well adapted organisms are. If adaptation is uncommon, and imperfect, natural selection may remain its proper explanation, but will be unimportant. The five authors here (Lewontin, Gould, Oster, E.O. Wilson, Maynard Smith) all agree that organisms are only imperfectly adapted, because the range of genetic variation available for selection is limited. The argument is strong, and not (so far as I

know) doubted by anyone; which is only unfortunate for Lewontin and Gould, who would discover an orthodoxy of "adaptationists" (all of whom believe organisms to be perfectly adapted), portray them in caricature, and analyse them with Marxist absurdities.

Sober remarks in his preface that it is difficult to distinguish the science from the philosophy in this anthology. For the most part I would agree; but only for the most part. In the sections that discuss whether theories are true, the science is indeed generally indistinguishable from the philosophy, and there are numerous scientific authors. The distinction could be made; but only superficially, by language and style (I could not help noticing the philosophers are more long-winded than scientists, but perhaps that is only the selection). Two sections, however, deal not with the validity, but with the nature, of theories — these are on functional explanation (teleology) and the reduction (or not) of Mendelian principles to molecular genetics — and they are entirely written by philosophers. Only, it would seem, when philosophical argument is used to criticize

theories do scientists become seriously interested.

The final section takes leave from the theory of natural selection. Mayr, and Sokal and Crovello, discuss the nature of species — whether reproductively ("biologically") or phenetically defined — and Hennig, Hull, Felsenstein and Farris discuss the three main schools of classification.

I often wonder who buys these anthologies. They are usually too expensive for individuals; but why on earth should a library buy a selection of papers it already possesses? I think *Conceptual Issues in Evolutionary Biology* may be an exception. Most of its contents originally appeared in philosophical journals that are not subscribed to by biological libraries; but they should all interest biologists who enjoy thinking about the theory of evolution. And every biological library in the world, I hope, eagerly serves that species of reader.

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Keeping cool

H.M. Rosenberg

Matter at Low Temperatures.

By P.V.E. McClintock, D.J. Meredith and J.K. Wigmore.

Blackie: 1984. Pp.258. Hbk £19.75; pbk £10.25.

IT IS time that we had a new introduction to low temperature physics, and this book fits the bill very satisfactorily. Each of the three authors is well-schooled in the cryo-arts and together they have produced a unified text in decent English, with the occasional felicitous turn of phrase that made me pause and smile.

The book assumes a background of undergraduate physics, but the first three chapters gently remind the older reader of certain essentials. For the final-year undergraduate and the beginning graduate student (by whom the book will probably be most used) they will act as a useful revision.

The authors begin by introducing the general principles of low temperature physics, with particular emphasis on the third law of thermodynamics, the effects of zero point energy and macroscopic quantization. The next two chapters deal with lattice vibrations and electrons, respectively, and in each case the differences between the behaviour of crystalline and of amorphous materials is well brought out. The meat of the book comes next in accounts of superconductivity, liquid helium 4 and liquid helium 3 (including the properties of mixtures of ^3He and ^4He). In each case a

simple phenomenological introduction is given as well as an outline of the current theory—with ample references for those who need a more detailed theoretical treatment. The final section of each of these chapters attempts to bring the reader up to date (to 1983) with developments in the most recent fields of interest—the properties of one-dimensional conductors and the search for other boson and fermion fluids.

The last two chapters cover more practical matters. Chapter 7 gives a good outline of experimental methods — ^4He and ^3He cryostat design, the dilution refrigerator, magnetic and Pomeranchuk cooling — and there is a helpful discussion on thermometry for various purposes. Finally, the authors turn to the applications of low temperature physics, the major emphasis, of course, being on superconductivity (high field electromagnets, the application of Josephson effects in computer elements, squids and other devices). This concluding chapter ends with a brief account of the uses of liquified gases themselves, including the interesting application of superfluid ^4He as a couplant in the acoustic microscope and as a non-reacting surface in the neutron bottle.

All in all, this is a good useful text. Not only that, it is enjoyable to read—an unusual and refreshing attribute. □

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New in paperback: Jonathan Slack's *From Egg to Embryo* published by Cambridge University Press, price £9.95, \$24.95. For review see *Nature* 306, 294 (1983).

New for neuroscience

With the meeting of the American Society for Neurosciences this week, we feature a range of products to interest the neurosciences expert, including a new assay for monitoring tricyclic antidepressants.

● A new HPLC assay that monitors the five major tricyclic antidepressants and active metabolites simultaneously in serum or plasma has been introduced by Waters Associates. The Waters HPLC assay of the tricyclic antidepressants (TCAs) simultaneously monitors amitriptyline, imipramine, desipramine, nortriptyline, doxepin and their clinically relevant metabolites in the same assay without cross reactivity. The assay has been evaluated for interference, correlation with GC and linearity and takes just 12 minutes. Full documentation is provided in a method book. The TCA Assay was designed for Waters QA-1 Analyzer, an HPLC system providing convenient push-button operation. With the same instrument, technicians can perform assays of other psychotropic agents such as tetracyclines, butyrophenones, phenothiazines, benzodiazepines and other therapeutic drugs.

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● The recent 'hole-poke' program developed for Columbus Instruments animal activity meters, monitors simultaneously eight animal cages equipped with 'hole-poke' boards. Each time an animal pokes its nose into one of the 16 holes located in the cage's floor, it interrupts two crossing infrared beams. Information about interruption is decoded by the Apple III computer which keeps track of the number of pokes in each hole, the time an animal spent with its nose in the particular hole, as well as totalized number of pokes and time spent 'poking' for each animal cage. The 'hole-poking' method is an established procedure for testing an animal's exploratory behaviour, a valuable indication of animal brain functions. Information collected by an Apple II computer is periodically printed and stored on the disc. The same system can be used to monitor animal feeding activity with feeders (food dishes) located underneath a 'holed' floor. When monitoring animals' feeding, computer results will correspond to numbers of visits to the feeders and time spent eating. Animal food preferences are easily established from computer records. 'Hole-poke' systems can be applied in neurotoxicology, experimental psychology, development of new drugs, as well as studying nutrition. Other Apple II computer programs from Columbus Instruments allow plotting of animal's movements, number of rearings, animal rotational behaviour and separation of stereotypic from ambulatory activity.

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● LDC/Milton Roy is pleased to announce a new interface package for the CI-10 computing integrator to an Apple II or Apple IIe computer. The new interface allows all CI-10 functions to be accessible from the Apple Keyboard. In addition, any CI-10 file (slice files, peak files or method files) can be loaded from the CI-10 to the Apple for further data manipulation or enhancement by the Apple. This additional capability of the LDC/Milton Roy CI-10 is designed for ease of use and provides the user with an independent chromatography integration channel for the Apple computer. Up to 15 CI-10 computing integrators can be networked from one IEEE board.

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● Two new models in its 700 Series laboratory XY recorder are being introduced by Allen Datagraph, Inc. Model 720 offers eleven ranges in X and Y and accepts Option 30, which provides digital control of the recorder and annotation of plots. Model 725 has eleven ranges plus a built-in time base. These new models extend the usefulness of the basic XY recorder in OEM and laboratory applications. The 700 Series' standard features include Pad-load paper supply, DC servo operation, sealed feed back potentiometers and low drift circuitry. These recorders offer fibre-tip pens, optional carrying case, English or metric calibration and a digital interface option.

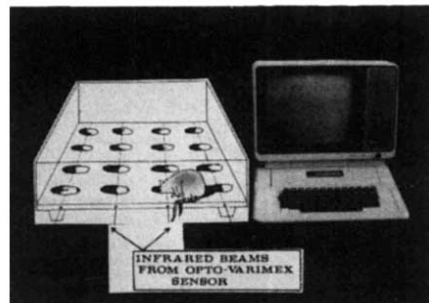
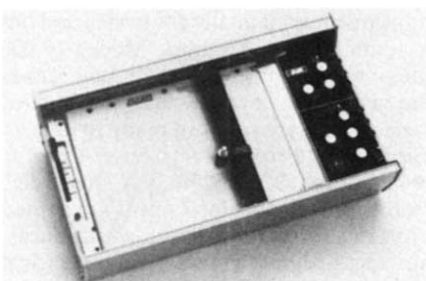
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● Wheaton Instruments announces the new Wheaton Biostir low profile heavy duty magnetic stirrer designed for use with Wheaton Celstir suspension culture flasks; it will accept one vessel up to 8 litres in size. The solid state speed control provides a speed range of 25 to 350 r.p.m. even with wide fluctuation in line voltage. The unit features a powerful alnico magnet for increased efficiency and the low profile cabinet conserves incubator space. For added protection, the circuit board has been coated with a special sealant so that the Biostir low profile stirrer can be used in high humidity incubators.

Circle No. 104 on Reader Service Card.

● Scientific Systems, Inc., now offers a pneumatically-actuated version of its 3XL Injection Valve, designed to permit automated and manual use of the valve. Available for both internal and external loop operation, the pneumatically-actuated 3XL provides load-to-inject times of 1/10 second at 50 psi. On the internal loop version, switching from one sample loop to another takes only 15 seconds. The valve has several unique design features that maximize efficiency. A straight flow path from the sample loop to the column prevents sample flow disruption and maintains a more symmetrical and efficient peak shape. To minimize band dispersion, the 3XL allows direct coupling of standard size columns to the valve. An event marker aids data handling.

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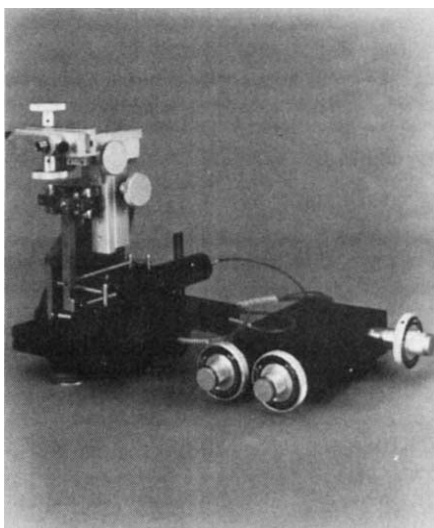
Allen Datagraph Model 720 recorder (top left), Wheaton Instruments' Magnetic Stirrer (top right), Waters' HPLC assay for monitoring antidepressants (bottom right) and 'hole-poke' program for monitoring animal behaviour from Columbus Instruments (bottom right).

● Dekasol from ICN Biomedicals Inc. is a powerful concentrated solution formulated for the rapid control of radioactive contamination. Dekasol removes radioactive particles from surfaces by a two-way action. It will sequester metallic ions which contaminate surfaces, and, because of its high detergent action, will lift and suspend contamination particles. The contamination is then easily rinsed away with water. For routine use, Dekasol is diluted 20 to 50 fold and can be used on virtually any type of surface; it is mild enough to be used on skin.

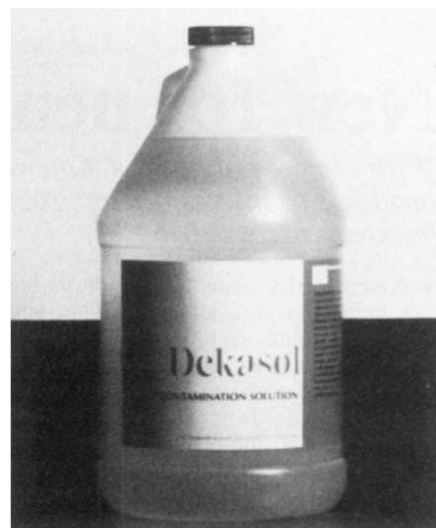
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● A new patch clamp stimulator is now available from the Medical Systems Corporation. This unit, model S-100, is a low-noise pulse generator which is specifically designed for use with extra cellular patch clamps. When functioning as a stimulator, the S-100 provides voltage pulses (jumps) to the patch membrane. These same pulses can also be used as test pulses to measure the resistance of the patch pipette. The S-100 can be operated in a "free running" mode, in which it provides repetitive pulses to the patch; or in a "single shot" mode, in which it is triggered manually or externally. The output pulses of the S-100 are always paired and the first and the second pulse have independently adjustable magnitude and duration. Both unipolar and bipolar output pulses are available. The Model S-100 also functions as a simulator of single channel activity with a pseudo-random channel generator included for control and simulation of patch activity. Medical Systems Corporation is a specialist in the development and marketing of instruments used in electrophysiology.

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World Precision Instruments' Series 10000 micromanipulator (left) and Dekasol solution for the control of radioactive contamination from ICN.



● From World Precision Instruments Inc. the Model DVC1000 Dual Voltage clamp provides continuous measurement of the transepithelial currents while voltage clamping two separate membranes (one membrane as a control and the second membrane as the test material) at zero volts or at a preset voltage difference. This procedure allows the study of membrane permeability and ion transport under different experimental conditions.

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● Hydraulic coupling of the fine control on World Precision Instrument Inc.'s new Series 10000 micromanipulators virtually eliminates vibration associated with touching the micromanipulator during adjustment. Hydraulic movements feature backlash-free operation and typically exhibit drift of less than 1 micron in a twenty-four hour period. Model 10000 can be fitted with one, two, or three hydraulic movements and offers 0.2 micron resolution for fine control. Coarse adjustment is manually controlled and has a resolution of 0.1 mm. Model 10,000 micromanipulators with hydraulic drives are available in right and left-handed versions and are supplied ready to use.

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● The new Series 200 X-Y Recorder, featuring Houston Instrument's patented capacitance rebalance position transducer, operates without the friction and electrical noise associated with slide-wire potentiometers. The Series 200 performs with high speed and accuracy and can meet the needs of both educational and industrial users, with applications ranging from materials testing to compound analysis. By flipping a switch, users may select one of eleven input ranges on pen (Y) and beam (X) axis, starting at 1mV/inch, or one of eight beam (X axis) speed levels, ranging from 200 seconds/inch to 1 second/inch. A slew rate of more than 30 inches/second ensures high fidelity recording of test and measurement data.

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● The Multipoint Recorder/Logger (MRL) from the Esterline Angus Instrument Corporation provides analog trend recording, digital (alpha-numeric) data logging or a combination of both and a comprehensive choice of user-programmable performance functions offered in a multipoint recorder/logger. Simplified, programming is accomplished through a colour-coded panel keyboard. Programmable features include: mode of operation, chart speed, scan interval, peak or valley detection, printout/output time, scale limits, engineering units, real time clock and calendar, and three alarm setpoints per channel in standard or failsafe mode.

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● A new microprocessor controlled osmometer, the Multi-Osmette from Precision Systems, performs freezing point determinations of osmolality on up to 24 samples. The new instrument accepts micro samples in disposable plastic tubes, calibrates automatically, makes the determination of osmolality, and with an optional printer, provides a fully annotated record for each sample. The complete cycle is activated by a single button. Automatic processing of multiple samples can free as much as an hour of technician's time, and a printed record eliminates questions of identification of results; use of inexpensive disposable plastic sample tubes eliminates the problems of glassware washing and breakage. When stat determinations are required, the automatic cycle can be interrupted. One or more stat samples can be introduced and run, after which the auto-cycle is resumed. Operation of the automatic sampler changer is simple, with easy operation for single samples when desired.

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